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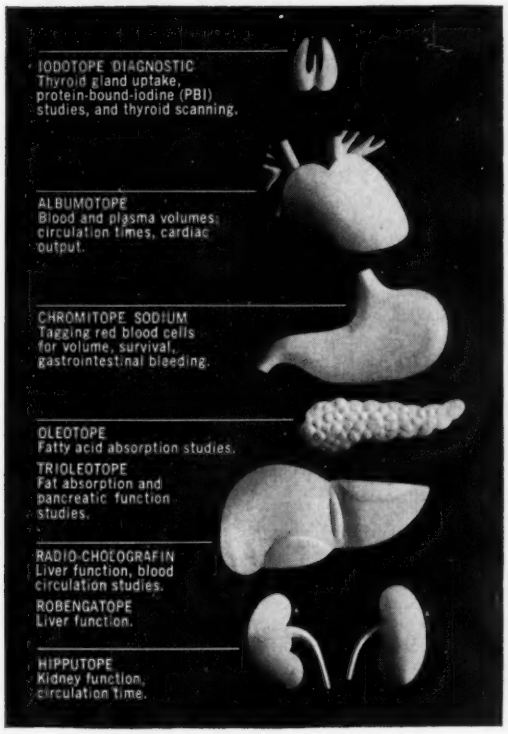

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DISEASE	NUMBER OF PATIENTS	RESULTS				INADEQUATE TRIAL
		GOOD	FAIR	TRANSIENT	FAILURE	
Lymphoma	**74**	**34**	**3**	**5**	**23**	**9**
Hodgkin's Disease	29	10	3	4	9	3
Lymphosarcoma	21	15	0	0	3	3
Multiple Myeloma	16	9	0	0	4	3
Reticulum Cell Disease	8	0	0	1	7	0
Leukemia	**23**	**10**	**0**	**0**	**8**	**5**
Chronic Lymphatic Leukemia	8	4	0	0	3	1
Acute Monoblastic Leukemia	11	5	0	0	3	3
Acute Myeloblastic Leukemia	4	1	0	0	2	1
Carcinoma (Breast, Lung, and Solid Tumors)	**29**	**2**	**1**	**1**	**23**	**2**
Miscellaneous (Mycosis, Fungoides, Psoriasis)	**4**	**0**	**0**	**1**	**3**	**0**
Total	130	46	4	7	57	16

Adapted from Wall, R. L., and Conrad, F. G.

Note that the neoplastic disorders most responsive to Cytoxan were lymphosarcoma, multiple myeloma, Hodgkin's disease, and chronic lymphatic leukemia. Occasionally, good results were observed in acute monocytic leukemia and carcinoma of the breast.

Other advantages noted in this study*

• multiple routes of administration, permitting prolonged maintenance therapy • lack of latency period for bone marrow depression • failure to produce significant thrombocytopenia • potential therapeutic effect in diseases usually unresponsive to other mustard compounds (*e.g.*, myeloma).

*Wall, R. L., and Conrad, F. G.: Arch. Int. Med. *108*:456-482, 1961.

INDICATIONS: Cytoxan is valuable for palliative therapy of certain malignant neoplasms, particularly some of those arising in the reticuloendothelial and hematopoietic systems and certain solid tumors.

Types of cancer which have proved relatively more susceptible or more resistant to Cytoxan therapy may be grouped as follows:

Group I: Neoplasms relatively susceptible to Cytoxan
- Hodgkin's disease
- Lymphomas: lymphosarcoma; giant follicular lymphoma; reticulum cell sarcoma
- Leukemia: acute; chronic
- Mycosis fungoides

Group II: Neoplasms relatively resistant to Cytoxan
- Malignant neoplasms of the breast and the ovary*
- Malignant neoplasms of the lung, the gastrointestinal tract and the genitourinary system, including the cervix and the uterus
- Malignant neoplasms of miscellaneous origin
- Malignant melanomas

*Malignant tumors of these organs are somewhat more susceptible to Cytoxan therapy than are the others included in this group.

DOSAGE: *For neoplasms relatively susceptible to Cytoxan* — Patients with lymphomas and other neoplasms believed to be relatively susceptible to Cytoxan therapy are given an initial dose of 2 to 3 mg./Kg./day intravenously. White blood counts and platelet determinations should be made daily or twice weekly and the dosage adjusted accordingly. Intravenous infusions should be continued for at least 6 days unless otherwise indicated. A leukopenia of between 1500 and 5000 cells per cu. mm. (or lower) may be expected between the tenth and fourteenth day. In the presence of a leukopenia of less than 2000/cu. mm. Cytoxan should be discontinued until the white cell count returns to 2000 to 5000 (usually within a week). Dosage is subsequently adjusted as indicated by the patient's objective response and the leukocyte count. If the patient is subjectively improved, if the size of the tumor has decreased, or if the white cells are satisfactorily maintained between 2000 and 5000/cu. mm. oral dosage may be instituted equivalent to intravenous dosage.

Thrombocytopenia is rarely observed on this regimen. If platelet counts of less than 100,000/cu. mm. are observed, the patient should be watched carefully. If platelets continue to decrease, Cytoxan should be discontinued.

The patient who has had previous treatment with alkylating agents, or x-ray, or is debilitated may be more susceptible to bone marrow depression, and initial Cytoxan doses should be more conservative than the above. Such patients should have more frequent hematologic evaluation. Good medical practice demands access to a reliable hematologic laboratory when using Cytoxan.

For neoplasms relatively resistant to Cytoxan — Patients with carcinomas and other malignant neoplasms believed to be less susceptible to Cytoxan therapy are given a dose of 4 to 8 mg./Kg./day intravenously. Unless there are indications to the contrary, this dose is continued for 6 days, then stopped. Leukopenia usually ensues on the tenth to fourteenth day after the first dose of Cytoxan. Thrombocyte reduction is not common, and platelets may actually increase. The leukocyte count promptly returns toward normal levels in most cases, and as it begins to increase, sufficient Cytoxan is administered to maintain it near 2000 to 5000/cu. mm. This may be accomplished by two intravenous injections weekly, or by oral administration, or by a combination of both routes. An oral dosage of 50 to 200 mg. daily or an intravenous injection of 5 mg./Kg. twice weekly will usually suffice.

The platelet and leukocyte counts should be followed carefully, and the prior treatment history of patients carefully evaluated as delineated above.

Leukopenia as a guide to adequacy of dosage — The best objective measure for dosage seems to be the number of circulating white blood cells. This is used as an index of the activity of the hematopoietic system, especially the bone marrow. The mechanism by which Cytoxan causes a reduction in the level of white blood cells is not known, but cessation of dosage results in an increase in the level, indicating that the hematopoietic system had not been permanently affected. When large doses (8 mg./Kg./day for 6 days) are given initially, the white cell count falls rapidly. Following the cessation of the 6-day course, the white cells may continue to decline for as long as 8 days and then increase. The reduction of the white cell count during Cytoxan therapy and its subsequent increase when therapy is discontinued can be repeated in the same patient. Maximal reduction in leukocyte count indicates the maximal permissible Cytoxan level for therapeutic effect. Leukopenic patients must be watched carefully for evidence of infection.

Total white blood cell and thrombocyte counts should be obtained 2 or more times weekly in order to evaluate therapy and to adjust dosage.

SIDE EFFECTS: Although Cytoxan is related to nitrogen mustard, it has no vesicant effect on tissue. It does not traumatize the vein when injected intravenously, nor does it cause any localized tissue reaction following extravasation. It may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally or directly into the tumor, when indicated. It is apparently active by each of these routes.

Nausea and vomiting are common and depend on dose and on individual susceptibility. However, many investigators accept the nausea and vomiting in favor of maintaining maximal therapy. The vomiting can be controlled with antiemetic agents.

Alopecia is a frequent side reaction to Cytoxan therapy. It has been observed in 28% of the patients studied in this country. The incidence is greater with larger doses. The loss of hair may first be noted about the 21st day of therapy and may proceed to alopecia totalis. This effect is reversed following discontinuance of Cytoxan; during reduced maintenance therapy, hair may reappear. It is essential to advise the patient in advance concerning this effect of the drug.

Dizziness of short duration and of minor degree has occasionally been reported.

Leukopenia is an expected effect and can be used as a guide to therapy. Thrombocytopenia may occur, especially after large doses. The leukocyte or platelet counts of an occasional patient may fall precipitously after even small doses of Cytoxan, as with all alkylating agents. The drug should be discontinued in such patients and reinstituted later at lower dosage after satisfactory hematologic recovery has occurred. Prior treatment with x-ray or with other chemotherapeutic agents frequently causes an earlier or exaggerated leukopenia or thrombocytopenia after Cytoxan medication. Only rarely has there been a report of erythrocyte or hemoglobin reduction.

ADMINISTRATION: Add 5 cc. sterile water (Water for Injection, U.S.P.) to 100 mg. of Cytoxan in the sterile vial (add 10 cc. to 200 mg. vial). Shake, allow to stand until clear, remove with sterile syringe and needle and inject.

The freshly prepared solution of Cytoxan may be administered intravenously, intramuscularly, intraperitoneally, intrapleurally, or directly into the tumor. The solution should be administered promptly after being made but is satisfactory for use for three hours after preparation.

If the patient is receiving a parenteral infusion, the Cytoxan solution may be injected into the rubber tubing if the solution is glucose or saline.

No thrombosis or thrombophlebitis has been reported from injections of Cytoxan. Extravasation of the drug into the subcutaneous tissues does not result in local reactions.

PRECAUTIONS: Cytoxan should not be given to any person with a severe leukopenia, thrombocytopenia, or bone marrow infiltrated with malignant cells. It may be given with suitable precautions to patients who have had recent x-ray treatment, recent treatment with a cytotoxic agent, a surgical procedure within 2 to 3 weeks, or debilitated patients.

AVAILABILITY: Cytoxan is available as follows:

Cytoxan for Injection, 100 mg., a sterile dry-filled vial containing 100 mg. cyclophosphamide and 45 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan for Injection, 200 mg., a sterile dry-filled vial containing 200 mg. cyclophosphamide and 90 mg. sodium chloride. Packaged, 12 vials per carton.

Cytoxan Tablets for oral administration, 50 mg., white, round tablets, flecked with blue for easy identification. Packaged, 100 tablets per bottle.

For a copy of the Cytoxan brochure, or other additional information on Cytoxan, communicate directly with the Medical Department, Mead Johnson Laboratories, Evansville 21, Indiana.

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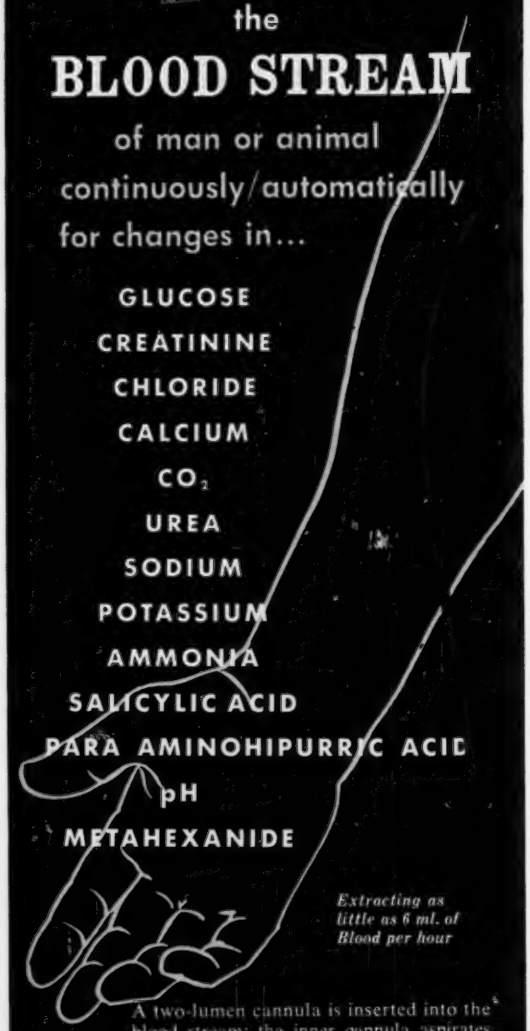

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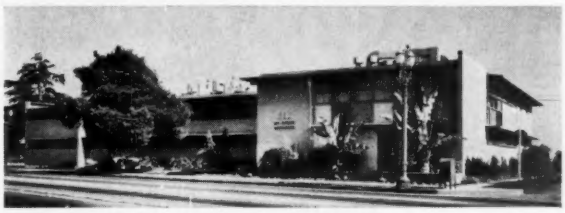

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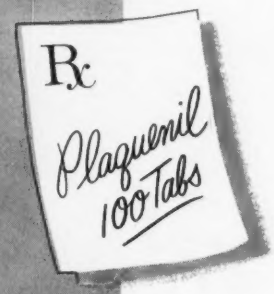

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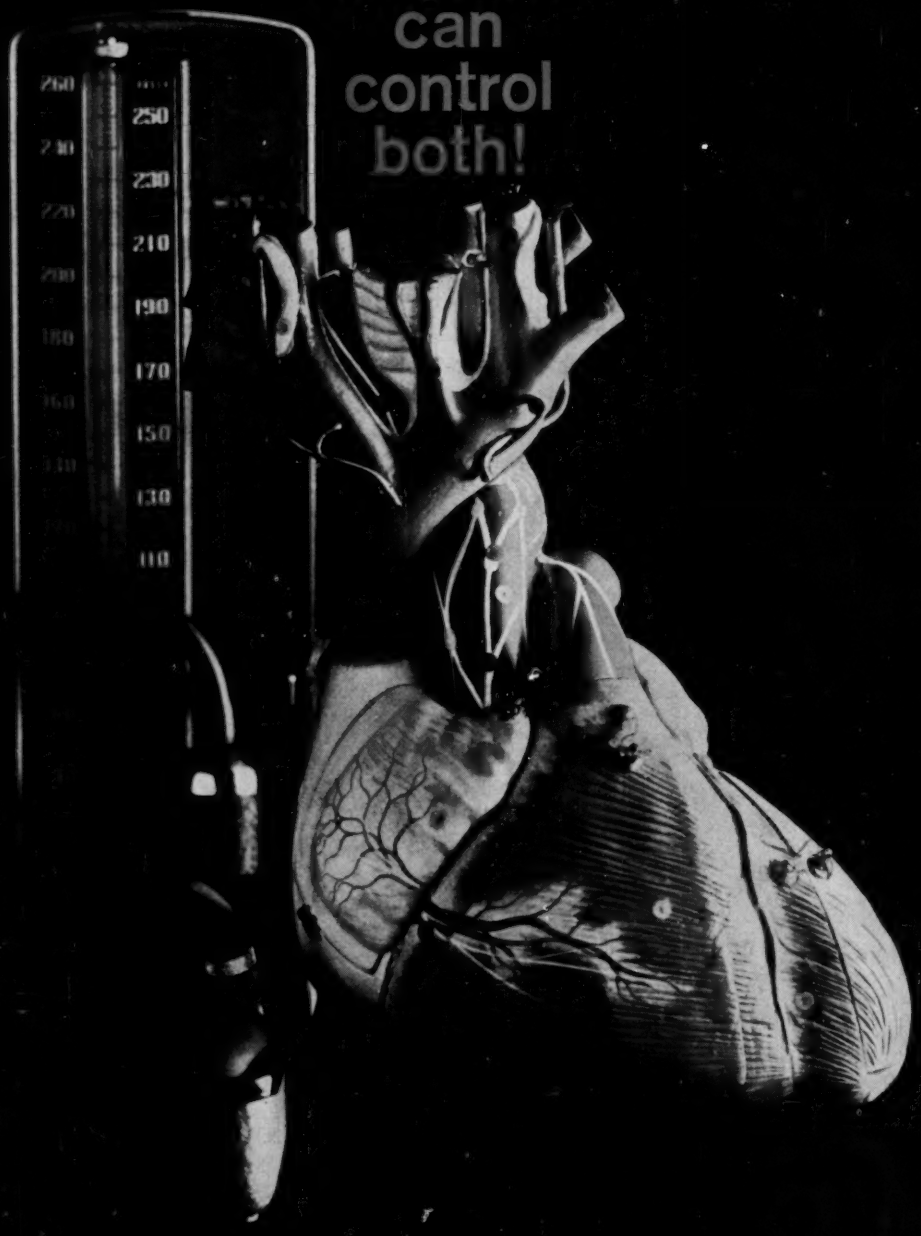

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Serpasil—in addition to its well-established effectiveness in controlling high blood pressure—offers an important bonus in treating hypertension. Laboratory studies show that Serpasil can prevent stress-induced heart damage,[1,2] presumably through its ability to deplete the catecholamines (epinephrine and norepinephrine) from the myocardium.[3,4]

These laboratory data are clinically significant in light of growing evidence[5-7] that more than purely "mechanical" overwork may be involved in cardiac damage associated with hypertensive disease. Raab[5] suggests that much of this damage is due to a direct metabolic action of the catecholamines on heart muscle. The way to prevent it, he believes, is to deplete or inactivate excess catecholamines.

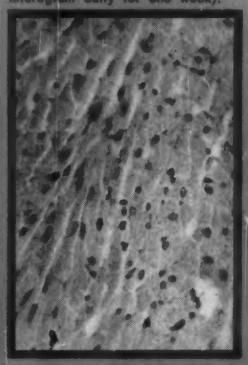
Thus, Serpasil not only eases the mechanical burden on the heart by reducing peripheral resistance and slowing heart rate, it may also provide protection against catecholamine-induced heart damage—the added benefit in prescribing Serpasil for hypertension.

References: 1. Raab, W., Stark, E., and Gigee, W. R.: Circulation 20:754 (Oct.) 1959. 2. Raab, W.: Research report to CIBA. 3. Carlsson, A., Rosengren, E., Bertler, A., and Nilsson, J.: Psychotropic Drugs (edited by Garattini, S., and Ghetti, V.), Elsevier Publishing Company, Amsterdam, 1957, pp. 363-372. 4. Waud, D. R., Kottegoda, S. R., and Krayer, O.: J. Pharmacol. & Exper. Therap. 124:340 (Dec.) 1958. 5. Raab, W.: Am. J. Cardiol. 5:571 (May) 1960. 6. Bayer, O., Borden, N. E., Boeminghaus, H., and Effert, S.: Ztschr. klin. Med. 146:607 (June) 1950. 7. Raab, W.: Hormonal and Neurogenic Cardiovascular Disorders, The Williams & Wilkins Company, Baltimore, 1953, pp. 407, 465.

LABORATORY EVIDENCE SHOWS SERPASIL PREVENTS STRESS-INDUCED HEART DAMAGE[2]

Severe heart damage in unprotected stressed rat. Tissue taken from rat given 2-α-methyl-9-α-fluorohydrocortisone and stressed (by restraint) for 18 hours. (Photomicrographs from Raab.[2])

No heart damage in stressed rat protected with Serpasil. Tissue taken from rat given 2-α-methyl-9-α-fluorohydrocortisone and stressed as at left, but also given Serpasil (0.4 microgram daily for one week).

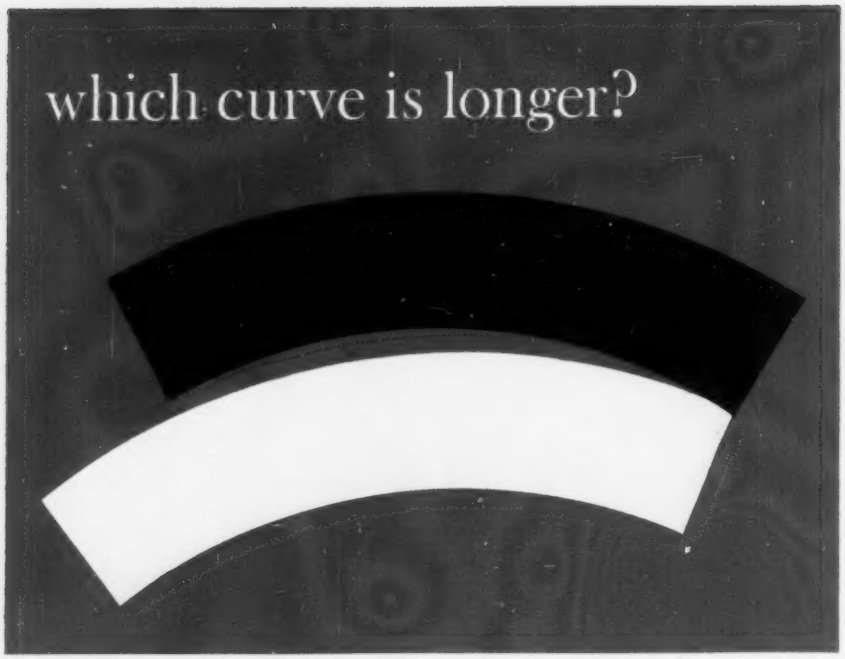


Note: While Serpasil did not completely protect the hearts of all animals in this study, it greatly reduced myocardial damage in most of them. Original magnification of photomicrographs: approximately 450 X.

Complete information about indications, dosage, cautions, and side effects of Serpasil—as well as a full report on its heart-protecting action—will be sent on request.

SUPPLIED: Tablets, 0.1 mg., 0.25 mg. (scored) and 1 mg. (scored).

Serpasil® CIBA Summit, N.J.



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PREPARATION AND BIOLOGICAL PROPERTIES OF A CHEMICALLY MODIFIED *ESCHERICHIA COLI* ENDOTOXIN OF HIGH IMMUNOGENIC POTENCY AND LOW TOXICITY

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The chemical basis of the toxic and immunogenic properties of endotoxins from enteric gram-negative bacilli has been the subject of intensive investigation. Recent methods of purification yielded biologically active preparations which were essentially free of dissociable protein and consisted of complex phosphorylated lipopolysaccharides containing firmly bound peptides (1, 2). Chemical degradation destroyed toxicity, liberating complex phosphoglycolipds and a haptenic polysaccharide endowed with serological specificity (1, 3–7). It was concluded that the lipid was essential for toxicity, whereas the polysaccharide merely functioned as a water-soluble carrier (7, 8). This concept was supported by the successful restoration of toxicity upon artificial recombination of the lipid with unrelated proteins (9, 10).

These findings suggested the possibility of preparing a nontoxic antigen by chemical dissociation of the lipid from the native endotoxin complex. In the past, all attempts to separate the toxic from the antigenic properties failed, since the hydrolytic methods employed lacked the required selectivity and invariably destroyed the antigenic properties more rapidly than the toxicity (1, 2). The use of nonhydrolytic reagents of greater specificity, on the other hand, appeared to offer a more promising approach. In the present study, the selective reduction of toxicity was accomplished by the treatment of a Boivin antigen from *Escherichia coli* with $LiAlH_4$. The data presented show that the resulting product retained the immunogenic potency and serologic activity of the original complex.

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† Supported by the Research and Development Division, Office of the Surgeon General, Dept. of the Army, Washington, D. C.

The choice of $LiAlH_4$ was suggested by earlier studies on the chemical structure of mycobacterial glycolipids. It has been shown that the fatty acids of these lipids are frequently linked to the carbohydrate portion through ester bonds which are reductively cleaved by $LiAlH_4$ without damage to the carbohydrate moiety (11–16).

MATERIALS AND METHODS

Reagents. $LiAlH_4$ and $NaBH_4$ were purchased from Metalhydrides, Inc. Pyrogen-free saline and distilled water were obtained from the Abbott Company.

Decontamination. Equipment (glassware, needles, and so on) was made free of pyrogen by heating at 170° C for 2 hours. Plastic material and other equipment that could not be exposed to this temperature were made pyrogen-free by immersion in 10 per cent NaOH for at least 2 hours and rinsed with pyrogen-free distilled water.

Bacterial cultures. *E. coli* of serotype 0:113, isolated from the blood of a patient, was subcultured on agar containing 5 per cent rabbit blood.

Media. For large scale growth, a synthetic salt medium, containing glucose as carbon and NH_4^+ as nitrogen source, was used (17).

Preparation of endotoxin. The bacteria were grown in 12.5-L batches as described previously (17). Growth was stopped after 18 hours by the addition of 75 ml of a 40 per cent formalin solution. The cultures were harvested with a DeLaval gyro-test unit, washed with 95 per cent ethanol, followed by acetone, and dried in a vacuum oven at 56° C. The bacterial mass, suspended in 5 parts of distilled water with a Waring Blendor, was incubated at 60° C for 90 minutes and subsequently at 4° overnight. An equal volume of cold 1.25 M CCl_3COOH was added with short mixing in a Blendor and the suspension kept at 4° C for 3 hours. The extract was separated by centrifugation at 12,000 rpm at 5° C, dialyzed for 48 hours against running distilled water, passed through a Seitz filter, and precipitated by the addition of 95 per cent ethanol to a final concentration of 68 per cent by weight. After 12 hours at 4° C, the precipitated endotoxin was collected, washed with 95 per cent ethanol followed by ether, dried *in vacuo* to constant weight, and stored over a desiccant. The endotoxin was obtained as a white powder in a yield of about 5 per cent based on the bacterial dry weight.

Chemical and spectroscopic assays. Carbohydrate was determined by the phenol-sulfuric acid method (18). Reference samples of glucose and endotoxin standard solutions were included in each assay. Fatty acid esters were determined by the method of Snyder and Stephens (19), as modified for endotoxins by Tauber (20). Crystalline tripalmitin served as standard.

Infrared spectra were recorded with a Beckman IR-4 double-beam spectrophotometer. The samples were prepared by the KBr micropellet technique as described previously (21).

Chemical treatment of endotoxin

Reduction with $LiAlH_4$. In a first experiment, a suspension of 300 mg of endotoxin in 30 ml of dry ether was refluxed for 5 hours. A total of 150 mg of $LiAlH_4$ was added in 10-mg portions at regular intervals. Excess reagent and the LiAl alkoxide addition products of the reaction were decomposed by careful addition of moist ether followed by 50 ml of water. Pyrogen-free distilled water was used throughout. The ether was separated and the aqueous phase re-extracted twice with 50-ml portions of ether. After drying over anhydrous Na_2SO_4, the pooled ether extracts were taken to dryness *in vacuo*, yielding 5 mg of a lipid residue. The insoluble $Al(OH)_3$ was removed from the aqueous phase by filtration and washed with water. The clear filtrate, after dialysis against water overnight and lyophilization, yielded 38 mg (13 per cent of the original starting material) of a white fluffy residue, designated "reduced endotoxin I" (RE I). The remaining major portion of endotoxin was firmly adsorbed to the aluminum hydroxide gel, since a carbohydrate assay of the resuspended precipitate accounted for the balance.

In a second experiment, the reaction mixture was immediately neutralized to pH 7 at the end of the reduction period in order to minimize the possibility of alkaline hydrolysis during the isolation of the water-soluble product. Thus, 50 mg of endotoxin in ether was refluxed with 100 mg of $LiAlH_4$ for 5.5 hours. The mixture was then decomposed by the addition of 3.1 ml 1 N HCl. After removal of the ether phase, the aqueous suspension was diluted to a volume of 20 ml and filtered through a fritted glass funnel of medium porosity. The filtrate contained 3.3 mg of reaction product, as determined by carbohydrate assay. It was deionized by passage through a column of Amberlite MB-3 mixed-bed resin which had been thoroughly washed with pyrogen-free distilled water. The lyophilized effluent yielded 3 mg residue, designated RE II.

Alkaline hydrolysis with LiOH. An aqueous solution of LiOH containing the equivalent of 10 mg $LiAlH_4$ per ml was prepared by decomposition of the hydride with water and filtration of the insoluble alumina gel. A series of tubes containing 50 mg of endotoxin and increasing amounts of LiOH in a total volume of 10 ml was allowed to stand at 20° C. The reaction was stopped by neutralization with the strongly acidic cation exchanger Amberlite IR 120 (H^+ form) and the product isolated by lyophilization. The resin was made pyrogen-free by previous treatment with 10 per cent NaOH followed by regeneration with 2 N HCl and extensive washing with pyrogen-free water.

Alkaline hydrolysis with NaOH. The preparation of NaOH-treated endotoxin is described below under "Antibody titrations by hemagglutination."

Bioassays

Pyrogenicity. The pyrogenic effect of the test preparations was measured rectally after injection into the marginal ear vein of carefully conditioned albino rabbits weighing 3 to 5 kg. Temperatures were recorded hourly for 8-hour periods prior to and after injection of the test materials. The experimental techniques and equipment used have been described in detail (22). Pyrogenic potency was measured by plotting the maximal temperature rise caused by endotoxin doses ranging from 0.0001 to 1.0 μg against the logarithm of the dose. The minimal pyrogenic dose, defined as the amount required to produce a 1.0° F (0.56° C) rise, was determined from the resulting linear log dose-response curve and gave a value of 0.00045 μg for the standard endotoxin preparation used in these experiments.

Lethal toxicity. Litter mates of white Swiss mice obtained from Flora O'Grady were injected into the tail veins with twofold dilutions of the test preparations. Groups of 5 mice were used for each dose and the toxicity expressed as the fraction of survivors after 72 hours. The LD_{50} was calculated by probit analysis according to Finney (23) in those cases where the data required a statistical test of significance.

Hematology. Hematologic changes were studied by total and differential leukocyte counts in duplicate on blood obtained from rabbits by nicking the central artery of the ear at zero time, 5 minutes, 1 hour and 4 hours.

Antibody titrations by hemagglutination. The concentration of antibodies against endotoxin preparations was determined by hemagglutination essentially as described by Neter and co-workers (24). One ml of saline containing 10.0 mg of endotoxin was added to 4.5 ml 0.25 N NaOH and incubated at 37° C for 3 hours. After neutralization with 4.5 ml 0.25 N HCl, 1.0 ml of packed human group O erythrocytes was added and the suspension incubated for 1 hour at 37° C. The red cells had previously been washed 4 times with saline. The sensitized red cells were separated by centrifugation, washed 3 times with saline and resuspended to a concentration of 2.5 per cent. Sera were inactivated by incubation in a water bath for 30 minutes at 56° C, absorbed with group O human cells to remove red cell agglutinins and diluted serially by adding 0.5 ml to 0.5 ml of saline. To each tube containing 0.5 ml of serially diluted serum was added 0.1 ml of the sensitized cell suspension and the mixtures incubated at 37° C for 60 minutes. After overnight refrigeration at 4° C, the tubes were shaken and the agglutination endpoint was determined by visual inspection of the state of dispersion of the red cell suspensions.

Antigenic analysis by precipitin reactions in agar

Precipitin reactions in agar were carried out by the diffusion technique of Ouchterlony (25) with sera of rats immunized with various endotoxin preparations.

Immunization of rats. Holtzman albino rats, weighing approximately 365 g, were divided into 5 groups and immunized according to the schedule in Table I. Two to 3 ml of blood was obtained by cardiac puncture at 4, 8, 15 and 30 days after the final injection. The blood was drawn from 2 rats each time in the first 3 groups and from 3 rats in the latter 2 groups.

Preparation of agar plates. Agar diffusion plates were prepared essentially as described by Halbert, Swick and Sonn (26). A solution of 0.7 g of Bacto-agar (Difco) in 100 ml boiling saline was mixed, after cooking, with 0.8 g sodium barbital and 7.5 g glycine and the pH adjusted to 7.4 with 0.1 N HCl. Eight ml molten agar was poured into petri dishes measuring 60 × 20 mm and allowed to solidify about a mold with one center and six outer holes of 8 mm diameter. The mold was removed from the hardened agar and the plates stored at 4° C for 1 to 4 days, allowing excess fluid to seep into the wells whence it could be removed by suction.

In one group of experiments, the test was performed by placing 0.2 ml antigen solution containing 200 μg of the test preparation in the center well and 0.2 ml of serum in the outer wells; in other experiments, various antigens were pipetted into the outer wells and the antiserum into the center well. Plates were incubated in the refrigerator and examined for precipitation bands 48 hours later and daily thereafter.

Immunization against E. coli-induced pyelonephritis in rats

Twenty male albino rats of the Holtzman strain, weighing about 365 g, were injected intraperitoneally with 1.0 μg $LiAlH_4$-treated endotoxin (RE I) and 2.0 ml blood was collected by cardiac puncture 7 days later from each of 5 rats. Since no hemagglutinating antibodies were detected, all rats were given a second injection of 19 μg of the same antigen and cardiac blood was drawn again from the previously bled 5 rats 7 days after the second injection. Antibody titers had risen sharply to titers ranging from 1 : 32 to 1 : 256. Ten days after injection of the second antigen dose, 2 ml of cardiac blood was drawn from all of the 20 immunized rats and from 20 nonimmunized controls. Immediately afterward, each rat was inoculated intracardially with 0.5 ml of an 18-hour culture in trypticase soy broth containing approximately 10^9 cells per ml of the same *E. coli* strain from which the endotoxin had been extracted. The kidneys of the animals were massaged for 5 minutes through the intact abdominal wall in order to produce pyelonephritis (27). Seven days later all surviving rats were sacrificed by exsanguination and exposure to ether. Their kidneys were examined for the pathological changes of pyelonephritis and the severity of the disease graded according to the system illustrated in Figure 1. Each kidney was then weighed and ground to a pulp in a homogenizing tube with a Teflon-tipped, motor-driven, stainless steel pestle. The individual homogenates were diluted serially in steps of 1 : 10 with water and pour plates made of each dilution. After incubation at 37° for 24 hours, the total number of viable bacteria per gram of tissue was calculated for each kidney from the colony counts.

TABLE I

Immunization schedule of rats for antigenic analysis

Group	No. of rats	Antigen	Amount	Route
1	6	Untreated Boivin endotoxin	20 μg*	i.p.
2	6	Reduced endotoxin, prep. RE I	20 μg*	i.p.
3	6	NaOH-treated endotoxin	20 μg*	i.p.
4	12	18-hour broth culture of heat-killed† *E. coli*	0.5 ml‡	i.c.
5	12	18-hour broth culture of viable *E. coli*	0.5 ml‡	i.c.§

* Two injections at 1-week interval.
† 80° C for 60 minutes.
‡ 10^9 Bacteria per ml.
§ Intracardial injection followed by renal massage.

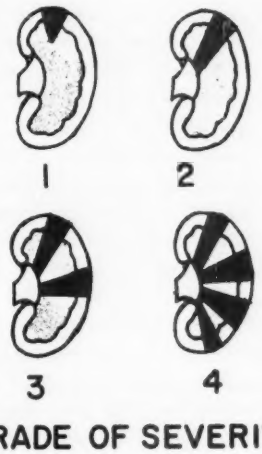

FIG. 1. QUANTITATIVE ESTIMATION OF SEVERITY OF PYELONEPHRITIS. Progressive stages are graded 1 to 4 as shown by the schematic drawings.

RESULTS

Physicochemical characterization of endotoxin preparations. It is well known that the chemical composition of endotoxin preparations varies considerably, depending upon the method of isolation, but under carefully standardized conditions, materials of fairly constant nitrogen, carbohydrate and lipid content are obtained. The Boivin type lipopolysaccharide-protein complexes, extracted with trichloroacetic acid and characterized by a

relatively high nitrogen content (2 to 4 per cent), are both toxic and immunogenic. Preparations of equal toxicity, but lower nitrogen content (1.0 to 1.5 per cent), have been obtained by various extraction and purification procedures, sometimes with loss of immunogenicity (1, 2, 9, 28, 29). Since the primary objective of this study was to reduce the toxicity of highly antigenic preparations rather than to purify for increased toxicity, a Boivin type endotoxin was used without further purification.

For the analytical characterization of the endotoxin preparations under study, quantitative infrared spectrophotometry and a sensitive colorimetric assay for carbohydrate (18) were used. Infrared spectroscopy has proved to be a most powerful analytical tool in investigations of complex lipids, particularly for a preliminary characterization of preparations of unknown purity and chemical structure (15, 16, 30). Webster, Sagin, Landy and Johnson have published infrared spectra of a Boivin antigen, a protein-free lipopolysaccharide, and degraded polysaccharide (hapten) which clearly show that the proportions of protein, carbohydrate and lipid in these preparations are reflected in the appearance and relative intensity of characteristic absorption bands (1).

The infrared spectrum of the Boivin antigen used in these studies (Figures 2, 4, 5) was highly reproducible from one batch to another and similar to the spectrum published by Webster and coworkers. The carbohydrate content (expressed as glucose equivalents) was constant within $\pm$ 5 per cent and corresponded to 32.5 per cent of the dry weight. Acid hydrolysis with constant boiling aqueous HCl in an atmosphere of nitrogen resulted in the maximal liberation of 4 per cent of $-NH_2$ after 90 minutes. Before hydrolysis, the content of free amino groups in endotoxin was 0.3 per cent, as determined with ninhydrin (31).

Effect of reduction with $LiAlH_4$ on chemical structure and biological properties of endotoxin. Treatment of endotoxin with an excess of $LiAlH_4$ gave a water-soluble reaction product and a small amount of an ether-soluble lipid. The water-soluble reaction product was a white powder which formed opalescent solutions in water. The carbohydrate content remained virtually unchanged, corresponding to 33.3 per cent before and 32.7 per cent after treatment.

The spectra of the original endotoxin and of the ether- and water-soluble reaction products are reproduced in Figure 2. The spectrum of the ether-soluble lipid (Figure 2b) shows the characteristic absorptions of a long chain aliphatic alcohol (15). Since ether-soluble long chain aliphatic alcohols have never been found in hydrolysates of endotoxin, they must have been derived from the corresponding fatty acids as a result of the reductive cleavage of ester bonds which originally linked the fatty acids to the carbohydrate or peptide moiety of the endotoxin. This conclusion is also supported by a spectral comparison of the water-soluble reaction product RE I with untreated endotoxin (Figure 2a). The most conspicuous changes are the decrease in the CH stretching absorptions near 2,900 cm^{-1} and the virtual disappearance of the CO stretching bands near 1,735 (C = O) and 1,250 (C – O) cm^{-1} in the spectrum of the water-soluble reaction product. No satisfactory explanation has been found for the appearance of a strong new band at 1,420 cm^{-1}. In the region of the strong carbohydrate absorptions between 1,100 and 1,000 cm^{-1}, the originally only faintly resolved band separates into two distinct peaks at 1,090 and 1,050 cm^{-1} after treatment. This effect may be explained by the elimination of a strong absorption band near 1,060 cm^{-1}, possibly due to the removal of organic phosphate, which is known to absorb strongly in this region. Significant modification of the carbohydrate moiety seems unlikely, since both carbohydrate content and the associated serological activity remained unchanged after treatment with $LiAlH_4$.

Biological properties. a) Toxicity. As is evident from the chart in Figure 3, intravenous injections of reduced endotoxin produced no fever or other signs of illness in rabbits, even in the highest dose of 100 μg tested. In contrast, the minimal pyrogenic dose of untreated endotoxin was 0.00045 μg, and doses of 5 μg were frequently lethal. Thus, treatment with $LiAlH_4$ reduced the pyrogenicity of endotoxin by a factor of at least 10^5.

The results of a toxicity assay in mice are shown in Table II. Intravenous injections of 1.0 mg of $LiAlH_4$-treated endotoxin failed to cause a significant lethal effect, but the available material was insufficient to permit an LD_{50} determination. In contrast, the LD_{50} of the original endotoxin was 0.10 mg.

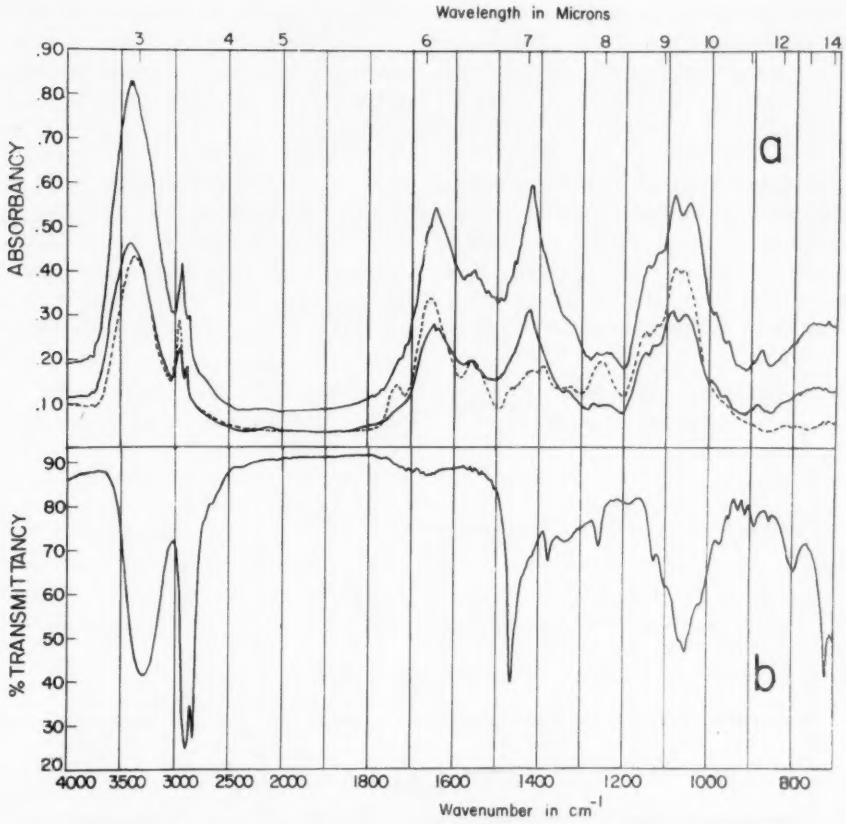

FIG. 2. EFFECT OF $LiAlH_4$ TREATMENT ON INFRARED SPECTRA OF ENDOTOXIN. a) Broken curve: untreated Boivin antigen, 27 μg per 4.3 mg KBr; solid curves: upper, nondialyzable water-soluble reaction product (RE I), 36 μg per 5.0 mg KBr; lower, 27 μg per 4.3 mg KBr. b) Ether-soluble reaction product (melt).

No significant change was observed in the leukocyte count of three rabbits during a 4-hour period after the intravenous injection of 5.0 μg of the ester-free endotoxin. Injection of the same amount of untreated endotoxin, on the other hand, produced a sharp neutropenia within 60 minutes, as was evident from the disappearance of 60 to 80 per cent of the neutrophiles from the circulation.

TABLE II

Lethality for mice of reduced and original endotoxin

Preparation	Fraction dead after 72 hours	
	RE I	Orig. endotoxin
mg inj.		
0.063	0/5	4/10
0.125	0/5	4/10
0.25	0/5	10/10
0.50	1/5	10/10
1.00	1/5	10/10
LD_{50} mg	≫1.0	0.10

A further manifestation of toxicity is the erythema caused by endotoxin injected into the human skin (32). Three human volunteers were injected intracutaneously with 0.1 μg of reduced endotoxin RE II into the right forearm and with an equal dose of regular endotoxin into the left forearm. The $LiAlH_4$-treated preparation caused no visible reaction, whereas all three subjects developed erythema and intense induration at the injection site of the regular endotoxin. This reaction began in 2 hours, reached a maximum in 12 hours and subsided in 48 hours.

b) Pyrogen tolerance. The results of tolerance studies, illustrated in Figure 3, show that the injection of the nonpyrogenic ester-free endotoxin (RE I, RE II) conferred tolerance to the untreated, pyrogenic parent preparation. The nonpyrogenic endotoxin was given according to three different injection schedules. Two groups of three rabbits received six consecutive daily intravenous injections of 1.0 μg (A–C) and 10.0 μg (D–F); a third group (G–I) received one injection of 100 μg. Seven days after the first injection, all nine rabbits were challenged simultaneously with six controls by the intravenous injection of 0.5 μg untreated endotoxin. All nine rabbits given prior injections of nonpyrogenic endotoxin responded to this challenge with typical, tolerant, monophasic fever curves, while the controls showed the usual biphasic fevers characteristic of the susceptible nontolerant state.

From the hemagglutinin titers recorded in Figure 3, it can be seen that the production of tolerance with the nonpyrogenic endotoxin was also accompanied by a significant rise in circulating antibodies prior to the date of challenge. It should

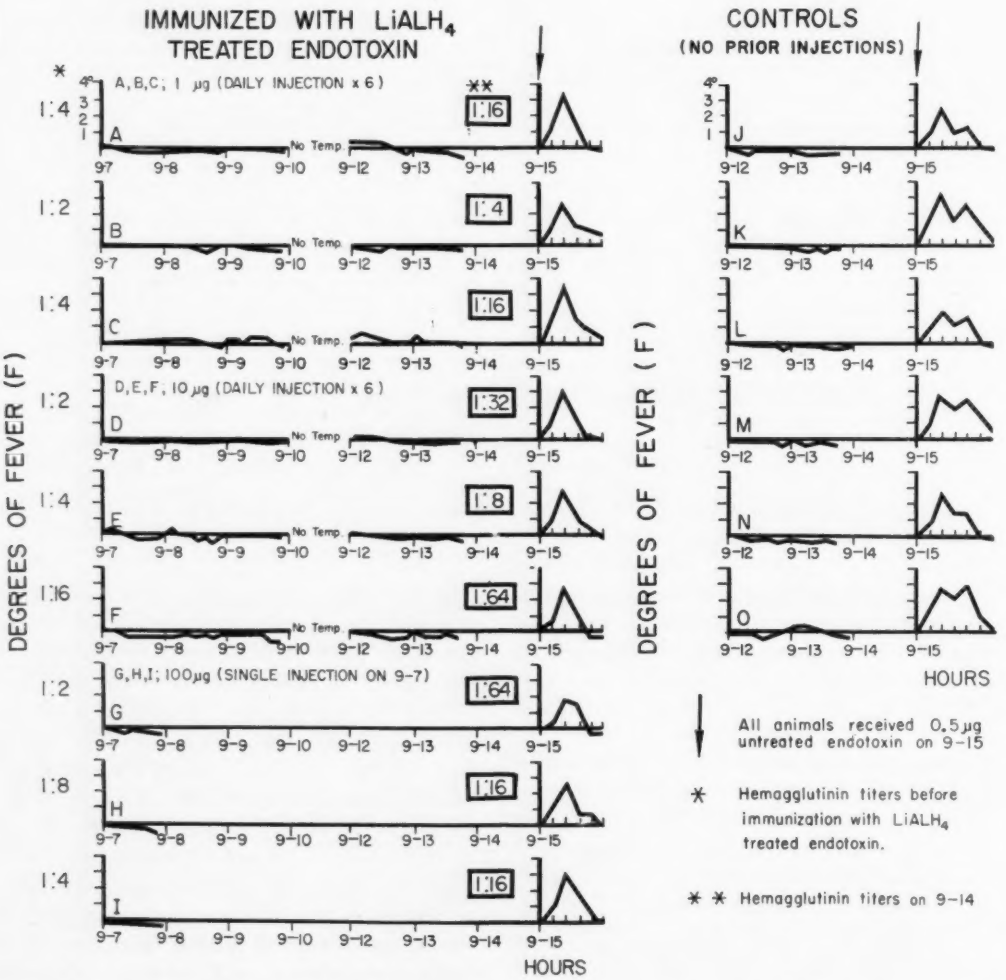
IMMUNIZED WITH LiALH4 TREATED ENDOTOXIN
CONTROLS (NO PRIOR INJECTIONS)
A, B, C; 1 μg (DAILY INJECTION x 6)
D, E, F, 10 μg (DAILY INJECTION x 6)
G, H, I; 100 μg (SINGLE INJECTION ON 9-7)
No Temp.
DEGREES OF FEVER (F)
HOURS
All animals received 0.5 μg untreated endotoxin on 9-15
* Hemagglutinin titers before immunization with LiALH4 treated endotoxin.
** Hemagglutinin titers on 9-14

FIG. 3. INDUCTION OF PYROGEN TOLERANCE IN RABBITS WITH NONPYROGENIC ENDOTOXIN PREPARED BY TREATMENT WITH $LiAlH_4$.

be pointed out that these titers reflect the degree of cross reaction with the NaOH-treated antigen required for sensitization of the red cells in this test and hence may not express the true antigenicity of the modified endotoxin for rabbits. The homologous antibody response could not be determined with this technique, since the $LiAlH_4$-treated endotoxin failed to adsorb to the surface of erythrocytes.

c) Tolerance to the lethal effect of endotoxin. The injection of nonpyrogenic endotoxin into mice also caused an increased tolerance against the lethal effect of subsequent challenge doses with regular endotoxin. The results obtained with two different preparations of $LiAlH_4$-treated endotoxin, listed in Table III, show that the LD_{50} of mice challenged 1 week after one injection of 1.0 μg of either treated or untreated endotoxin increased about threefold over that of the controls. The LD_{50} values of the immunized animals and the controls were computed by probit analysis and the difference was found to be statistically highly significant ($p < 0.01$).

TABLE III

Increased resistance of mice against lethal toxicity of endotoxin 7 days after immunization with one injection of 1.0 μg of regular and $LiAlH_4$-treated endotoxin

Challenge dose	Nonimmunized controls	Fraction dead after 72 hours: Immunized, regular endo.	Immunized, $LiAlH_4$-treated endo.*
mg			
0.063	4/10	1/10	0/10
0.125	4/10	3/10	1/10
0.25	10/10	1/10	2/10
0.50	10/10	8/10	9/10
1.00	10/10	10/10	10/10
LD_{50}† mg	0.10	0.29	0.31

* Half the mice were immunized with preparation RE I, the other half with RE II. Statistical considerations showed that pooling of the data was permissible and desirable.
† Computed by probit analysis (23).

Influence of reaction time and stoichiometry of reagents. A few experiments with varying proportions of reagent were carried out in order to obtain some information on the stoichiometry and time course of the reaction. Samples of 10 mg endotoxin were treated with 0.1, 1.0 and 100 mg of $LiAlH_4$ for 1 hour in the usual way. The reaction was stopped by the addition of water, the aqueous solutions neutralized, and adjusted to a volume of 5 ml. The insoluble hydroxides were removed by centrifugation and the supernatants

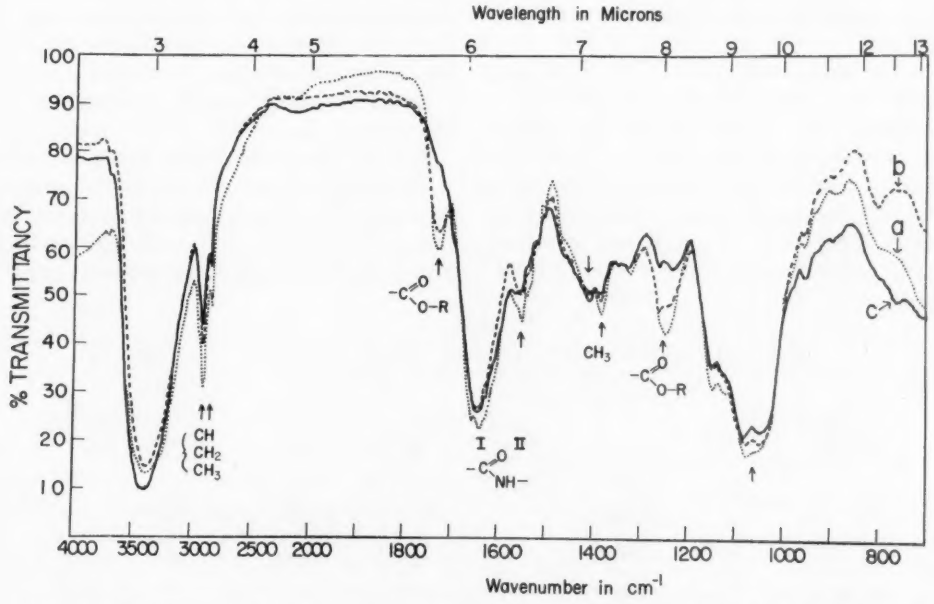

FIG. 4. CHANGES IN INFRARED SPECTRUM OF ENDOTOXIN AFTER TREATMENT WITH VARIOUS PROPORTIONS OF $LiAlH_4$. a) Untreated endotoxin. b) Treated with 0.1 mg $LiAlH_4$ per 10 mg endotoxin. c) Treated with 1.0 mg. Samples were pressed into 5 mg KBr pellets containing 36 μg endotoxin.

TABLE IV

Reduction of pyrogenicity and recovery of reaction product after treatment of endotoxin with various proportions of $LiAlH_4$ and for different reaction periods

Preparation	Endotoxin	$LiAlH_4$	% $LiAlH_4$ of endo. wt	Endo. adsorbed to $Al(OH)_3$	mg adsorbed / mg $Al(OH)_3$	Reaction period	Pyrogenicity in % of untreated endo.	IR spectrum
	mg	*mg*		*%*		*hrs*		*Figure*
RE III	10	0.1	1	16		1	~100	4b
RE IV	10	1.0	10	40	2.0	1	~1	4c
RE I	300	150	50	87	0.9	6	<0.001	2a
RE II	50	100	200	93	0.23	5.5	<0.001	*
RE V	10	100	1,000	96	0.05	1	†	†

* Similar to Figure 2a (not shown).
† Not tested because of lack of material.

(RE III, IV, V) assayed for carbohydrate. Spectroscopic and pyrogenicity determinations were carried out with the residues obtained after lyophilization of the deionized aqueous solutions RE III and Re IV. The results of this and the previous two experiments, summarized in Table IV, show that both efficiency of inactivation and adsorption losses increase with increasing amounts of reducing agent per milligram of endotoxin. If the reagent exceeds about 50 per cent of the weight of the endotoxin, the adsorption losses approach 90 per cent. Near saturation—i.e., with an excess of endotoxin—the aluminum hydroxide gel can bind about twice its weight of endotoxin (RE IV).

The effect of increasing proportions of reducing agent on the chemical structure of endotoxin is best visualized by comparison of the infrared spectra of the preparations listed in Table IV. The spectral changes reflecting various degrees of reduction are illustrated by the three superimposed recordings in Figure 4. It can be seen that treatment with even the smallest proportion of $LiAlH_4$ (0.1 mg per 10 mg endotoxin), which failed to produce a measurable decrease in pyrogenicity, caused a marked reduction, corresponding to about 25 per cent of the absorptions associated with ester groups (curve b). Further increase of the reducing agent to 1 mg $LiAlH_4$ per 10 mg endotoxin gave a product of about 100-fold reduced pyrogenicity, which shows only a trace of ester carbonyl at 1,735 cm^{-1} (curve c). Chemical determination (20) of the ester content of the untreated endotoxin gave a value of 0.27 μmole ester linkages per mg, corresponding to an ester-bound fatty acid content of 5 to 10 per cent. The finding that, after reductive cleavage, only about 2 per cent ether-soluble lipid was recovered in the form of aliphatic alcohols suggests that the major portion was adsorbed to the aluminum hydroxide gel.

The analysis of the stoichiometry and kinetics of the reaction is complicated by the presence in endotoxin of a large proportion of labile hydrogen which reacts rapidly with hydride to form complexes that are later decomposed with water.

TABLE V

Effect of treatment with LiOH on toxicity and CO stretching absorption of endotoxin

Preparation	Endo.	LiOH concentration		Reaction period	LD_{50}*	Toxicity	Absorption intensity at 1,250 cm^{-1}
			$LiAlH_4$ equiv.				
	mg	*moles/L*	*mg/10 ml*	*hrs*	*mg*	*%*	*%*
	50	0	0	0	0.098	100	100
HE I	50	0.005	2	1	0.176	56	85
HE II	50	0.01	4	1	0.211	46	64
HE III	50	0.025	10	1	0.324	30	48
HE IV	50	0.1	40	1			48
HE V	50	0.25	100	1	0.522	19	48
HE VI	50	0.25	100	48			48

* Estimated by the method of Reed and Muench (34).

This side reaction consumes a relatively large proportion of reagent. Replacement of labile hydrogen, most of which is associated with the carbohydrate portion, is calculated to require an amount of $LiAlH_4$ corresponding to 10 to 20 per cent of the endotoxin weight. Reductive cleavage of all ester bonds, on the other hand, would consume only 0.5 mg $LiAlH_4$ per 100 mg of endotoxin.

Subsequent experiments indicated that reductive cleavage of ester bonds alone fails to explain the observed loss of pyrogenicity. When the formation of insoluble $Al(OH)_3$ was prevented with sodium potassium tartrate (33), or when the reduction was carried out in aqueous solution with $LiBH_4$ or $NaBH_4$, only moderate reduction of toxicity occurred, despite the disappearance of the ester carbonyl band at 1,735 cm^{-1} in the infrared spectra of the reaction products. Apparently, the toxic products remaining after reduction with $LiAlH_4$ are preferentially adsorbed to the aluminum hydroxide gel. This was demonstrated by dividing the reduced reaction mixture into two portions, one of which was decomposed with dilute HCl as usual, the other with a saturated solution of sodium potassium tartrate. The fraction of the reaction product of the first portion which did not adsorb to $Al(OH)_3$ was free of pyrogenicity, whereas the product isolated from the tartrate solution had retained its toxic properties.

Alkaline hydrolysis. Endotoxin was treated with various concentrations of lithium hydroxide, in order to distinguish the chemical and biological effects caused by the reducing action of $LiAlH_4$ from those that are attributable to the hydrolytic action of its water-soluble alkaline decomposition product.

The LiOH concentrations and reaction periods are listed in Table V, together with the results of infrared spectrophotometry and mouse toxicity tests. The spectra of the samples treated with increasing concentrations of LiOH are reproduced in Figure 5. The results show that alkali causes both a reduction of toxicity and hydrolysis of ester bonds. The extent of hydrolysis after 1 hour, as measured by the intensity decrease of the ester bands near 1,735 and 1,250 cm^{-1}, was dependent upon the alkali concentration up to about 0.02 M and reached a value of about 50 per

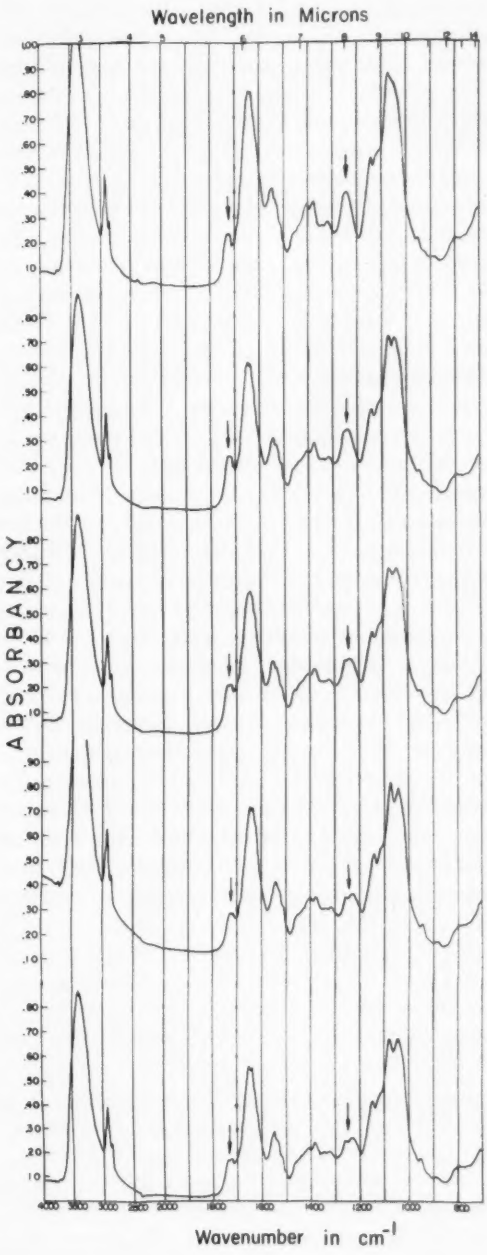

FIG. 5. CHANGES IN INFRARED SPECTRUM OF ENDOTOXIN AFTER TREATMENT WITH INCREASING CONCENTRATIONS OF LiOH, CORRESPONDING TO (FROM TOP) 0, 0.005, 0.010, 0.025, 0.25 M. Arrows indicate ester absorptions. (KBr pellets, 5 mg, containing 36 μg endotoxin.)

cent; an increase of the LiOH concentration to a final value of 0.25 M failed to cause further reduction of the ester absorption bands even after 48 hours' reaction time, as is evident from the infrared spectrum in Figure 5, curve 5. The reduction of toxicity as a function of the alkali concentration is shown in Figure 6. The LD_{50} is proportional to the log of the LiOH concentration, a tenfold increase of the latter producing an LD_{50} increase of 0.21 mg. Spectroscopic analysis, on the other hand, shows no difference between the spectra of the samples treated with 0.025 M LiOH for 1 hour and with a tenfold higher alkali concentration for 24 hours. Because of the lack of a strict quantitative correlation, it is doubtful that the moderate reduction of toxicity caused by alkali is directly related to the hydrolysis of ester bonds. This conclusion is in agreement with previous observations which indicated that alkaline hydrolysis under comparable conditions resulted in partial removal of lipid without significant reduction of pyrogenicity and toxicity (24).

Treatment of endotoxin with either LiOH or $LiAlH_4$ is accompanied in each case by an increasing separation of the broad absorption near 1,060 cm^{-1} into two distinct bands. However, in addition to these changes, alkali treatment, in contrast to reduction with $LiAlH_4$, also causes an increased separation of the amide I and II bands. This effect seems to be associated with the most alkali-sensitive portion of endotoxin, since it is caused by the lowest LiOH concentration tested (0.005 M) and is not noticeably enhanced at higher concentrations. This suggests that the observed spectral change results from a modification of the protein or peptide moiety which is known to adsorb in this region. This explanation is supported by the observed reduction of immunizing power of alkali-treated endotoxin preparations and the well known sensitivity of proteins to conditions of high pH. These data emphasize again the contrast between the lability of the immunogenic properties of endotoxin and the resistance of its toxicity toward alkali.

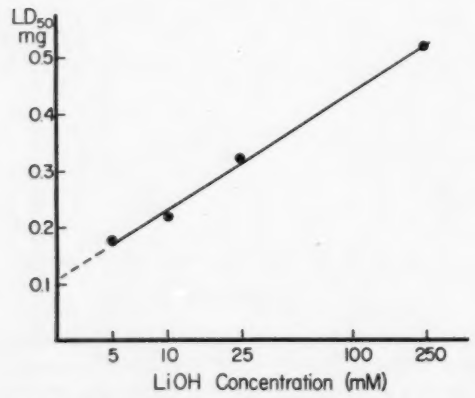

FIG. 6. LINEAR INCREASE OF LD_{50} FOR MICE WITH LOG OF LiOH CONCENTRATION AFTER TREATMENT OF ENDOTOXIN FOR 1 HOUR AT 22° C.

Antigenic analysis. In order to obtain further information on the immunizing properties and antigenic composition of various endotoxin preparations, rats were immunized and their sera analyzed for precipitating antibodies by the agar diffusion technique. Antisera produced with live and heat-killed *E. coli* cultures, as well as with Boivin antigen before and after treatment with $LiAlH_4$ and NaOH were each tested against four different antigens. The sera were collected at intervals over a 30-day period in order to follow the time course of the immune response. A complete synopsis of this experiment, showing the precipitin patterns obtained with the various antisera placed in the center and the antigens in the outer wells, is reproduced in Figure 7. Although all preparations were antigenic, the antibody response which they elicited varied considerably with regard to time, quality and intensity. The appearance of more than one band indicates chemical heterogeneity of the antigen preparations; by this test, autoclaved culture filtrates and untreated Boivin antigen contained at least two components, while the $LiAlH_4$- and alkali-degraded products contained three. So-called reactions of identity, characterized by the complete joining of the precipitin bands, occurred when different antigen preparations were tested against a given antiserum as well as in the reversed test. Analysis of the identity reactions indicated that all antigen preparations had at least one serologically reactive configuration in common.

The most rapid formation of completely cross-reacting antibodies was induced by the nontoxic, $LiAlH_4$-treated preparation (RE I). Sera obtained 4 days after the second injection of this antigen reacted with two precipitin bands against all antigens; after 8 days the inner bands began

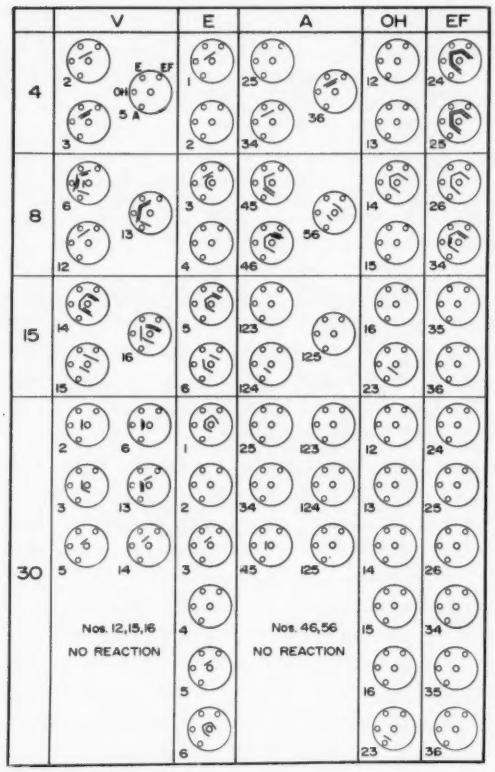

FIG. 7. DEVELOPMENT OF PRECIPITATING ANTIBODIES IN SERA OF RATS IMMUNIZED WITH VARIOUS ENDOTOXIN PREPARATIONS AS DEMONSTRATED BY THE OUCHTERLONY AGAR DIFFUSION TECHNIQUE. Antisera, prepared by the antigens shown at the top of each column, were placed in center wells and tested against the four antigens in the outer wells as indicated by the code. The sera were collected from individual rats 4, 8, 15 and 30 days after the second immunizing injection as indicated in the vertical column at left. The identification number of each immunized rat is placed to the left of each diffusion plate. Antigens: V = viable *E. coli* (18-hour broth culture containing 10^9 organisms per ml); E = untreated endotoxin; A = autoclaved *E. coli* (18-hour broth culture autoclaved for 2.5 hours at 120° C and 15 lbs pressure); OH = NaOH-treated endotoxin; EF = $LiAlH_4$-treated endotoxin RE I.

to disappear, and by 15 days no precipitating antibodies were detectable. A different pattern was shown by the antibody response resulting from immunization with either live *E. coli* or untreated endotoxin. In these cases, the antibodies appearing after 4 days gave precipitin reactions with unheated culture filtrates of *E. coli* and native Boivin antigen only. After 8 days, the antisera reacted with the alkali-treated endotoxin and with the soluble antigens of the autoclaved *E. coli* cultures and, finally, after 15 days with the $LiAlH_4$-treated antigen. This suggests that the native antigens are slowly degraded *in vivo* to products of increasing immunochemical similarity with the chemically modified test antigens. It is interesting that the chemically modified preparations could still induce the formation of antibodies capable of reaction with the original antigens, whereas the early antibodies elicited by the native antigens failed to precipitate with the modified antigen. This nonreciprocal cross reaction may be explained by the concept of competition of antigens as extended to different antigenic regions within the same molecule (35): the determinant region (epitype [1]) *a* which was immunogenically dominant in the original antigen must have been lost during treatment with $LiAlH_4$, thus allowing in the modified antigen the immunogenic expression of the previously repressed epitype *b*.

Other modifications of the immunogenic properties caused by detoxification are reflected in the greater intensity and shorter duration of the antibody response. The disappearance of antibodies within 15 days after the second immunizing dose suggests that the detoxified antigen is more rapidly metabolized than is the native endotoxin which stimulated the formation of antibodies for more than 30 days.

Alkali treatment, in contrast to reduction with $LiAlH_4$, greatly decreased the immunizing potency of the Boivin antigen, as indicated by the large proportion of rats which failed to respond and by the transient appearance after 8 days of only single precipitin bands against all antigens. Serological activity, on the other hand, was retained, since the NaOH-degraded product reacted strongly with the development of up to three bands against immune sera prepared with the other antigens. The alkali-treated haptene also showed a strong affinity for the surface of human erythrocytes, as demonstrated first by Neter and associates (24) and confirmed by the results of the hemagglutination tests in these experiments. In

[1] General adoption of the new terminology proposed by Jerne (36) is recommended, because it avoids present ambiguities and permits a more concise formulation of immunological concepts.

TABLE VI

Protection of rats against renal infection with E. coli after immunization with $LiAlH_4$-treated endotoxin

	Rats, n	Deaths after infection	Average weight at		Geometric mean* of number N of viable *E. coli* per g kidney at sacrifice	Log SD
			Infection	Sacrifice		
	no.	*no.*	*g*	*g*		
Immunized	20	0	366	371	1.0×10^5	1.76
Nonimmunized	20	3	357	334	25×10^6	0.71

* Antilog $[(\Sigma \log N)/n]$.

contrast, when red cells from the same source were exposed to the $LiAlH_4$-treated antigen under identical conditions, they could not be agglutinated by rabbit antisera which had a titer of 1 : 1000 with respect to erythrocytes sensitized with alkali-degraded endotoxin. This finding may be explained by the concept that lipid-lipid interactions play an essential role in the attachment to red cell surfaces (37).

Immunization of rats against pyelonephritis. The results reported in the previous sections show that immunization of animals with the nontoxic antigen provided protection against the toxic effects of *E. coli*. It was interesting to test whether this protective effect was also directed against the infection per se. The usual methods of determining the resistance against lethal challenge doses of live *E. coli*, however, fail to provide a measure of antibacterial immunity, since, under these conditions, death results primarily from the action of endotoxin. Experimental pyelonephritis, on the other hand, provides a more specific measure of infection by *E. coli* (38).

The results, summarized in Tables VI–VIII, show that immunization of rats with the nontoxic antigen (RE I) provided effective protection against experimental pyelonephritis comparable with that observed after immunization with the untreated parent endotoxin (39). The group of immunized animals gained weight and none of the rats died during the 26 days of the experiment, whereas the control group lost weight and three deaths occurred within 48 hours after the challenge infection. The geometric mean of the average bacterial count in the kidneys was reduced 25-fold in the immunized group (Table VI). A more detailed analysis of these findings is summarized in Table VII, in which the individual kidneys of the control and immunized group are ranked according to their viable bacterial count per gram of tissue. The resulting frequency distribution shows a striking difference between the two groups. Thus, 45 per cent of the kidneys of the immunized rats contained less than 10^5 viable bacteria per g of tissue compared with only 3 per cent in the nonimmunized controls. It is inter-

TABLE VII

Protective effect of immunization with detoxified endotoxin in E. coli-infected rats

Class interval	Bacteria/g kidney tissue*	Nonimmunized controls			Immunized rats		
		Kidneys	Accumulative	Av. grade pyelonephritis	Kidneys	Accumulative	Av. grade pyelonephritis
	no.	*no.*	*%*		*no.*	*%*	
X	3.2×10^7	1	100	1.0	0		
IX	1.0×10^7	4	97	1.3	5	100	1.4
VIII	3.2×10^6	10	84	2.2	2	87	2.0
VII	1.0×10^6	9	53	2.8	3	82	1.7
VI	3.2×10^5	3	25	1.3	8	74	1.1
V	1.0×10^5	4	16	2.2	3	53	1.0
IV	10^4	1	3	2.0	7	45	1.1
III	10^3	0	0		6	26	0
II	10^2	0	0		1	11	0
I	0	0	0		3	8	0

* Numbers in this column represent the lower limits of each class interval; e.g., class IV is defined by bacterial counts falling within 10^4 and 10^5 bacteria per g tissue.

esting to note that, in addition to the reduction of the mean bacterial count, immunization also causes a larger spread of the distribution, which is reflected in a more than twofold increase of the standard deviation, calculated on the basis of a log normal distribution with respect to the bacterial counts (Table VI). A similar effect has been observed in chemotherapeutic studies (40).

A comparison of the bacterial counts with the extent of pyelonephritis (Table VII) shows no strict parallelism between these two manifestations of infection. Whereas kidneys containing fewer than 10^4 bacilli per g were free of pyelonephritis, the kidneys with the largest lesions did not show the highest bacterial counts. This observation is consistent with earlier findings which showed that, during the course of infection, the kidneys tend to become sterile, while the tissue destruction often continues in the absence of viable bacilli (38).

Table VII further shows that immunization greatly reduced the severity of pyelonephritis even in those kidneys which contained the same number of viable bacilli as the nonimmunized controls. This is revealed more clearly by ranking the infected kidneys according to the extent of the lesions. The resulting frequency distribution (Table VIII) shows that over 90 per cent of the controls had lesions of all severity grades, with a peak frequency of grade 2. In contrast, 50 per cent of the immunized group was free of pyelonephritis, and the remaining kidneys showed considerably less involvement than the controls.

The persistence of pyelonephritis in the absence of viable *E. coli* suggests that the inflammatory reaction may be largely due to the endotoxin formed in the kidney during the infection. The reduction of pyelonephritis, which was observed when kidneys of immunized rats were compared with controls containing a similar number of viable bacteria (Table VII), is consistent with this interpretation, since immunization with detoxified endotoxin has been shown to increase the resistance against the toxic effects of the original endotoxin. The resulting immunity, however, was also directed against the bacterial cells, as is obvious from the smaller number of viable bacteria present in the kidneys of the immunized rats. Since endotoxin is a major constituent of the bacterial surface and is known to produce antibodies capable of bacterial agglutination, the more rapid elimination of viable bacteria in immune animals may be readily explained by classical immunological mechanisms, such as enhanced phagocytosis. The lack of congruence shown by the frequency distributions of the different criteria used for the quantitative measurement of protection suggests that antibacterial immunity is a rather complex phenomenon which develops by a number of separate and at least partially independent mechanisms.

TABLE VIII

Reduction of E. coli-induced pyelonephritis in rats immunized with detoxified endotoxin

Extent of lesions	Nonimmunized controls		Immunized rats	
	Kidneys	Accumulative	Kidneys	Accumulative
grade	*no.*	*%*	*no.*	*%*
4	4	100	1	100
3	7	88	5	97
2	15	68	7	85
1	5	24	7	68
0	3	9	20	50

DISCUSSION

The present study describes the preparation of a nonpyrogenic Boivin antigen of high immunogenic potency. All of the toxic phenomena tested —i.e., pyrogenicity for rabbits, lethality for mice, and induction of neutropenia in rabbits and intradermal inflammation in man—were greatly reduced or abolished by a chemical procedure which did not seem to cause extensive fragmentation of the molecule. The conclusion appears justified, therefore, that a single molecular configuration is responsible for all of these toxic manifestations. It further follows that integrity of the molecular structures necessary for toxicity is not an essential requirement for immunogenic activity. Moreover, the successful separation of these two biological activities made it possible, for the first time, to study the immunizing and protective activities of endotoxin without interference from the effects of toxicity.

Chemical basis of toxicity. A definite identification of the chemical structures responsible for toxicity has not been achieved, mainly because the lack of sufficient material prevented an extensive chemical characterization of the detoxified product. While it was consistently found that only

preparations that were free of all detectable ester carbonyl were nonpyrogenic, the crude ester-free reaction product was still highly toxic. In order to obtain a nonpyrogenic material, subsequent partitioning on aluminum hydroxide gel was necessary. This step apparently resulted in the adsorptive removal of toxic fractions. The elimination of ester-bound fatty acids by $LiAlH_4$ may therefore not be directly related to the process of detoxification.[2] It should also be pointed out that a portion of the fatty acids in endotoxin is bound through amide linkages (42) which are not split by $LiAlH_4$. With the exception of carboxy-esters, there is no evidence for the presence in endotoxin of groups that are known to be readily reduced with $LiAlH_4$. The infrared spectra of the reduction products indicate that most of the peptide linkages remained intact; acetal and glycosidic bonds in carbohydrates have generally been found resistant against this reducing agent (43). This is also supported by the observed preservation of immunogenic potency and serological specificity. It has not been determined, however, whether $LiAlH_4$ is capable of cleaving phosphodiester linkages.

Degradation studies with alkali failed to show a direct quantitative relationship between rate and extent of hydrolysis of ester bonds and reduction of toxicity. At the highest concentration tested (0.25 N) LiOH caused only a 50 per cent ester hydrolysis and a fivefold reduction of lethality for mice. Immunogenicity, on the other hand, was almost completely destroyed, whereas serological activity, in agreement with previous observations (3, 24), was preserved. These results suggest that under mild conditions, alkali degrades endotoxin primarily by attacking bonds other than ester linkages, most likely by hydrolysis of certain peptide bonds.

The problem of the chemical basis of toxicity has been a matter of debate. From experiments with artificial complexes, Westphal concluded that the lipid A portion was responsible for toxicity (7), whereas Ribi and co-workers and Tauber, impressed by the apparent lack of a correlation between toxicity and lipid content of different preparations (2, 20), attributed toxicity to the polysaccharide moiety (2). The present results, while not providing decisive evidence for either view, tend to favor the idea that toxicity is the result of certain physicochemical properties characteristic of the entire molecule, rather than the autonomous property of one of its components.

Antigenic analysis. Further information on the biological properties of endotoxin was obtained from immunization studies. The rat was chosen as the most suitable experimental animal, because it responded more uniformly with the formation of precipitating antibodies against small doses of endotoxin than does the rabbit. It should be emphasized that the wider use of the rabbit for immunological studies and its superior response to certain other antigens does not *eo ipso* make it a more acceptable test system for immunization experiments with endotoxin.

Antigenic analysis by the Ouchterlony technique revealed the presence of two major components in all preparations tested. It is interesting that, even with an extensively purified Boivin antigen of low nitrogen content, Landy, Johnson, Webster and Sagin found three reactive components against rabbit antiserum in Oudin's agar gel diffusion test (3). So far, no endotoxin preparations have been shown to be immunochemically homogenous by such tests.

The immunochemical relationship between the various antigens used for immunization and the effects of chemical treatment on the antigenic structure were most strikingly reflected in the changing cross reactivity of the antibodies during the course of immunization. The precipitating antibodies appearing early in the sera of rats immunized with live or autoclaved *E. coli* cultures or with native endotoxin reacted only with the latter antigen but not with the alkali- or $LiAlH_4$-treated preparations. This illustrates the close similarity of the Boivin antigen with the antigenic structure of the bacterial surface. The antibodies elicited by the detoxified preparation, on the other hand, immediately gave strong cross reactions with all other antigens, as is evident from the appearance of two concentric and completely joined band systems.

[2] Johnson (41) described the preparation from *Shigella sonnei* of a protein-lipocarbohydrate antigen which was devoid of lethal toxicity for mice and protected mice against lethal challenge doses of the parent organism. Although the chemical relationship of this material to the toxic O-antigens is not known, this finding suggests that the normally toxic O-antigen preparations might be partitioned into toxic and nontoxic fractions even without prior chemical modification.

This is contrary to the loss of reactivity toward the parent antigen usually observed with antibodies prepared against chemically modified antigens (44). Of considerable interest was the observation that the reaction of the native Boivin antigen with its homologous antiserum consistently produced only one precipitin band during the observed 30-day immunization period, despite the presence of a second antigenic component capable of precipitation with the sera from rats immunized with the detoxified antigen. These results indicate that the specificity of the antibodies elicited by the native Boivin antigen is mainly directed against those molecular structures that are modified during the process of detoxification.

In contrast to the extensive elucidation of the structural features of the polysaccharide moiety responsible for the O-antigenic serological specificity (45), little is known about the factors determining the immunogenicity of endotoxin. The well documented loss of immunizing power after exposure of endotoxin to phenol suggested that the dissociable protein is essential for immunogenicity (7). This explanation is contradicted by the finding that the immunogenic potency of Boivin antigen increased during removal of associated protein by methods other than phenol dissociation. Subsequent treatment of the "protein-free" preparation with phenol, however, destroyed the immunizing properties (1). The poor immunogenicity of phenol-treated lipopolysaccharide preparations was confirmed during the present investigations. Thus, antisera of rats, immunized with Westphal's highly purified lipopolysaccharide, failed to give any visible reaction with homologous or heterologous antigen when tested by the Ouchterlony technique together with known reference samples and under the same conditions as described in Materials and Methods. Immunization of rabbits with Westphal's preparation, on the other hand, produced a significant rise in hemagglutinin titers. Nevertheless, the potency of the native Boivin antigen was still considerably greater than that of the phenol-extracted lipopolysaccharide when the two preparations were compared on an equal weight basis in the rabbit.

Protective immunization. It is well established that immunization of animals with endotoxin increases their resistance against the toxic effects of subsequent challenge doses of endotoxin as well as against infection with the corresponding or related bacteria. The mechanisms responsible for these effects are often called nonspecific and, as implied by the term *tolerance,* are thought to reflect a nonimmunological change in host susceptibility brought about by the toxic properties of the molecule. The present findings do not support this notion, since most of the protective effects of endotoxin—e.g., pyrogen tolerance, and increased resistance to lethal toxicity as well as to infectious pyelonephritis—have now been produced with a chemically modified antigen of greatly reduced toxicity. Rather, these results seem to favor an immunological interpretation of the observed tolerance. Other observations that may be cited in support of this view are the passive transfer of tolerance with serum from tolerant animals (46), the tolerant fever responses observed after injection of mixtures of endotoxin with high titer homologous antiserum (47), the rapid rise of antibody titers in response to minute doses of endotoxin (3), and the linear relationship between the O-agglutinin titers of rabbit sera and their potency (ED_{50}) to protect mice against lethal challenge doses of the homologous organism (*Salmonella typhosa*) (3, 48).

The preparation of effective, nontoxic vaccines against gram-negative bacteria has been an unsolved problem of great practical importance. An interesting new approach was the recent preparation by Lüderitz, Westphal, Staub and Le Minor of an artificial antigen with colitose as the determinant group (49). Goats immunized with this preparation elicited antibodies capable of agglutinating certain strains of *E. coli.* It would be interesting to know whether these antigens are also capable of providing immunological protection against the toxic effects of endotoxin and against infection. The approach reported here appears to be of practical value, since it has been shown that chemical modification of a therapeutically interesting antigen may serve to improve its desirable properties, such as immunogenic potency, range of cross reactivity and freedom from toxicity. Efforts are now being made to determine the optimal conditions of detoxification, to standardize the reproducibility of the procedures and to increase the yields of the detoxified antigen for further chemical studies and for vaccination trials in man.

SUMMARY

The preparation of a nonpyrogenic Boivin antigen by reductive treatment with $LiAlH_4$ is described. Infrared spectroscopic studies showed that this procedure resulted in the elimination of ester-bound fatty acid.

The chemically modified product showed virtually no toxicity when tested for lethality in mice ($LD_{50} > 1.0$ mg) and no pyrogenicity in rabbits (0.1 mg); it also failed to cause leukopenia in rabbits (5 μg) or intradermal inflammation in man (0.1 μg).

Comparative studies showed that reduction with $LiAlH_4$ and controlled hydrolysis with LiOH gave products of different chemical and biological properties. Mild alkaline hydrolysis, while virtually abolishing immunogenicity in rats, caused only moderate reduction of the pyrogenicity for rabbits and lethal toxicity for mice.

Immunization of rabbits with the detoxified material induced typical pyrogen tolerance, and immunization of mice caused the same increase of resistance to lethal challenge doses of regular endotoxin as was observed after immunization with the parent endotoxin.

The formation of precipitating antibodies after immunization of rats with various endotoxin preparations was studied by Ouchterlony's agar gel diffusion technique. The sera of rats immunized with the detoxified product produced the earliest, strongest and most completely cross-reacting precipitin bands of all of the antigens tested.

Significant protection of rats against *E. coli*-induced pyelonephritis was observed after immunization with the nontoxic antigen.

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EFFECT OF UREA ON URINE CONCENTRATION IN THE RAT

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Urea is the principal end-product of nitrogen metabolism in mammals and is excreted almost entirely through the kidney. Studies by Shannon (1, 2) in the dog and by Chasis and Smith (3, 4) in man have shown that the renal clearance of urea is less than the inulin or creatinine clearance at all rates of urine flow, and that the clearance of urea increases as the urine flow rises. These observations are consistent with the hypothesis that urea is filtered at the glomerulus and that a portion of this filtered load diffuses out of the nephron as the fluid passes from glomerulus to renal pelvis.

During the past few years a considerable amount of evidence has accumulated that urea has a unique role in the renal concentrating mechanism. The maximal urinary concentration achieved during antidiuresis is reduced by a low protein diet (5–8). The addition of urea to such a low protein diet will return the maximal urine concentration to near normal levels. In medullary tissue slices removed from the kidneys of antidiuretic dogs, it has been shown that urea is one of the principal solutes (9–11). Levinsky, Davidson, Berliner and Eden (11, 12) have proposed a mechanism by which urea, accumulated in the inner medulla passively, is able to add to the urine osmolality. During the passage of tubular fluid along the collecting duct, the urea concentration rises progressively as water moves into the medullary interstitial space along the osmotic gradient created by the active transport of sodium in the medulla. The rising urea concentration in the collecting duct establishes a concentration gradient along which urea moves into the inner medulla. The urea which diffuses into the inner medulla serves to balance an equiosmolal amount of urea in the collecting duct, leaving most of the medullary sodium free to balance other osmotic constituents in the urine. It has recently been demonstrated that the permeability of the collecting duct to urea is increased by vasopressin (13) and that the concentration achieved in the medullary tissue by this mechanism depends upon the fact that the collecting duct is less permeable to urea than to water (14, 15). This hypothesis is consistent with the concept proposed by Chasis and Smith and by Shannon, that urea is handled passively by the kidney.

The possibility that urea excretion is regulated at the tubular level has recently been re-examined by Schmidt-Nielsen (16). It has been shown that the regulation of urea excretion in the camel (17) and sheep (18, 19) is dependent largely upon the animal's requirements for protein and only partially upon the plasma urea level, the glomerular filtration or the urine flow. Thus, the sheep on a low protein diet reabsorbs a larger fraction of the filtered urea than does the same animal on a normal protein intake.

Studies by Gamble, McKhann, Butler and Tuthill (20) have shown that, by replacing sodium chloride with an equiosmolar amount of urea in the diet of rats, the volume of urine required to excrete a given solute load is reduced. However, as the volume of urine decreased in their experiments, so did the water intake. Thus, it is impossible to determine whether the drop in urine volume preceded or followed the decrease in water intake. Crawford, Doyle and Probst (21) have re-investigated the effect of urea on urine concentration in rats. In an attempt to avoid the problem of interpretation encountered in Gamble's work these authors gave their experimental animals a daily injection of vasopressin. They demonstrated that in rats on a low protein diet urea supplements decreased the urine volume and increased the urinary non-urea solute concentration. The decrease in urine volume might again be attributable to a primary effect on water intake. However, the effect of urea supplements on the non-urea solute concentration suggests that urea may influence the active transport mechanisms of the renal tubule.

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The studies reported here were done to re-evaluate the role of urea in the renal concentrating mechanism of the rat. It has been confirmed that urea supplements given to rats on a low protein diet increase the ability of rat kidneys to concentrate the non-urea solute of the urine, and it is further shown that rats with a low urine urea concentration, whether produced by a low protein diet or by a mannitol infusion, can have a significantly higher urea concentration in the renal papilla and inner medulla than in the urine.

METHODS AND MATERIALS

Rats were maintained on an artificial diet (22) modified to contain a low protein (5.8 per cent casein) and high salt (1.6 per cent sodium chloride) content or on Purina rat chow for at least 10 days before the experiments were begun. Rats receiving urea supplements were fed the low protein diet described above with 1.8 per cent urea added. Food and water were given *ad libitum*. No measurement of intake was made, but the body weight of almost all the rats was constant or showed a slow increase while on the diet. Vasopressin when used was given intramuscularly as Pitressin tannate in oil (Parke, Davis; lot 302), 500 mU daily.

Two kinds of experiments were performed. In the first group 24-hour urine collections were obtained in special cages constructed to immobilize the rat horizontally on wide mesh wire. In this way urine could be collected with no contamination by feces, food or water and yet rats could be allowed food and water. Urine was collected under mineral oil and toluene. At the end of every 24-hour collection period, urine was expressed from the bladder of each rat until the urinary bladder was no longer palpable. For tissue analysis, rats were sacrificed by exsanguination and the kidneys immediately removed. A slice was cut from the center of each kidney perpendicular to a line joining the poles of the kidney and placed between Parafilm in a refrigerator at + 4° C. These slices were carefully dissected under a magnifying lens, weighed, and homogenized in water for analysis. Figure 1 is a schematic representation of the anatomy of the rat kidney and the approximate division between the various slices as dissected in these experiments. Less than 5 minutes elapsed between removing the kidney and homogenizing the slices. Tissue homogenates were not boiled or centrifuged but were promptly analyzed for urea or frozen until the analyses could be performed.

In the second group of studies experiments were performed by collecting urine from the ureters of anesthetized rats. Animals on a low protein diet as well as chow-fed rats were used. Under ether anesthesia, the abdomen was opened and a piece of no. 10 polyethylene catheter placed in one ureter. Upon completion of three or more collection periods, the kidney was removed, sliced as before, and analyzed for urea. Some rats on each diet received infusions of 15 per cent mannitol at rates varying from 0.25 to 4.8 ml per hour for 0.5 to 3.5 hours before samples were collected.

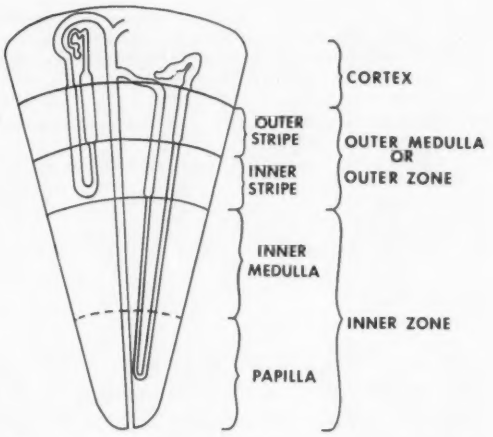

FIG. 1. A SCHEMATIC REPRESENTATION OF THE ANATOMY OF THE RAT KIDNEY TO INDICATE THE POSITION FROM WHICH VARIOUS SLICES WERE REMOVED.

Urea determinations were performed on tissue and urine by the microdiffusion method of Conway (23) and were corrected for any ammonia present by subtracting an ammonia blank. Sodium and potassium concentrations were determined with flame photometry (Baird-Atomic) with a lithium chloride internal standard. Osmolality was determined by freezing point depression (24). Tissue water was determined by drying slices from 3 groups of 2 rats each and averaging the values. All tissue concentrations were corrected for these water contents. The non-urea solute concentration of the urine was approximated by subtracting the determined urea concentration from the osmolality estimated from the freezing point.

RESULTS

The first set of experiments was performed to measure the effect of urea supplements on the urea and non-urea solute excretion of rats on a low protein, high salt diet. After 10 days on this diet collection periods were begun. There was considerable variation in the non-urea solute concentration achieved in consecutive collections from the same rat. In spite of this variation, however, it can be seen in Table I that five of the six rats receiving urea supplements were able to concentrate the urinary non-urea solute above 1,400 mOsm on at least one occasion. In 16 of the 29 collections from these rats the non-urea solute concentration was greater than 1,300 mOsm. In sharp con-

TABLE I

Effect of urea supplements on the non-urea solute concentration (NUSC) in 24-hour urine collections from rats on a low protein diet

With urea supplements			Without urea supplements		
Rat	Highest NUSC	Number collected	Rat	Highest NUSC	Number collected
A	1,462	4	a	1,156	4
B	1,521	5	b	1,214	5
F	1,557	5	c	1,084	2
H	1,339	6	d	1,301	4
K	1,411	5	h	1,130	5
V	1,614	4	t	1,162	6
			l	1,172	5
Average	1,484			1,174	

trast are the data from the seven rats not receiving urea supplements. Only one rat in this group had a non-urea solute concentration as high as 1,300 mOsm, while the others had non-urea solute concentrations at or below 1,214 mOsm on all occasions. Urea supplements thus enable rats on a low protein diet to increase the maximal non-urea solute concentration of their urine. These findings are in agreement with the observations of Gamble and co-workers (20) and Crawford, Doyle and Probst (21).

Tissue analysis. The results of tissue analysis from two groups of rats are shown in Figures 2 and 3. The urine values for sodium and urea plotted on the right-hand side of each figure were determined on 24-hour urine collections, ending at the time the animal was sacrificed. In Figure 2 the tissue values for sodium and urea in six normal rats not receiving exogenous vasopressin are presented. In these animals there is an in-

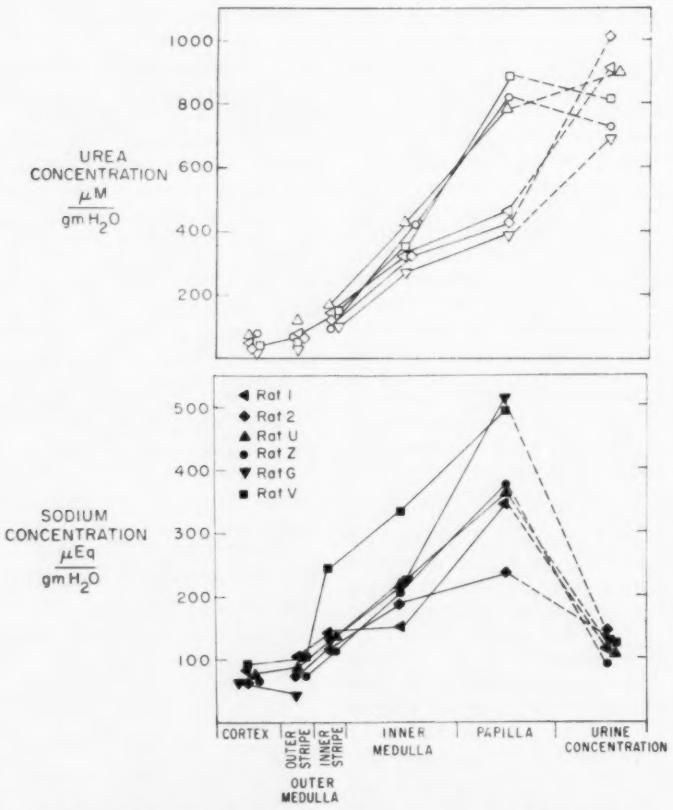

FIG. 2. TISSUE AND URINE ANALYSES ON SIX NORMAL RATS FED A CHOW DIET PRIOR TO URINE COLLECTION AND SACRIFICE. The upper frame contains the data for urea in tissue and urine, the lower frame the corresponding data for sodium. The concentration in μEq per g of water or per ml is plotted against the position of the tissue slice from which the analyses were obtained.

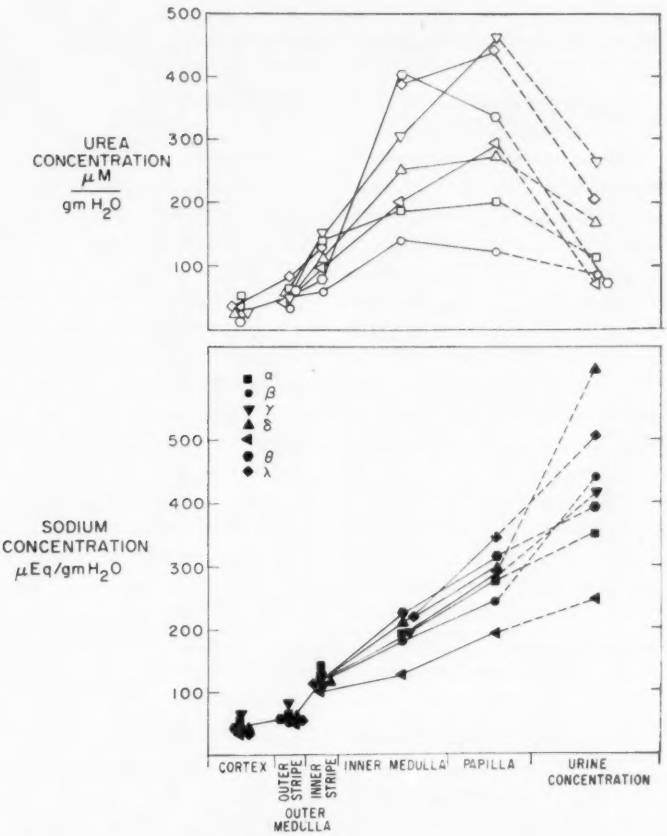

FIG. 3. TISSUE AND URINE ANALYSES FROM SEVEN PROTEIN-DEPLETED RATS. The upper frame contains the urea data, the lower frame the sodium data. The tissue concentrations per g of water are plotted against the position of the slice from which they were obtained.

crease in the tissue sodium concentration between the cortex and papilla, and in all cases the urine sodium concentration is less than the papillary sodium concentration. The tissue urea concentration also rises progressively from the cortex through the papilla. In all of these rats the urine urea concentrations are as high as or higher than the papillary urea concentration. A similar group of experiments was performed on six normal rats given vasopressin with essentially the same results.

The results of analyses of tissues from the rats on the low protein diet without urea supplements are shown in Figure 3. The rise in sodium concentration from cortex to papilla resembles that of normal rats, but in contrast with the previous analyses from normal rats, the urine sodium concentration is higher than the tissue sodium concentration. The tissue urea again shows the rise in concentration from cortex to inner medulla but only a small rise from inner medulla to papilla. In two rats the urea concentration in the inner medulla is actually higher than the papillary urea concentration. Even more striking is the fact that the urine urea concentrations are less than the papillary urea concentrations in all seven animals. The implication of these data and data from 16 other protein-depleted rats, which showed essentially the same findings, is that the rat on a low protein diet is able to accumulate urea in the medulla at a concentration up to two or three times greater than that in the urine.

TABLE II

Urea concentration in the urine and papilla of rats maintained on a low protein diet

Rat	Urine* flow	Urine urea	Papillary urea	Papillary urea	Papilla / Urine	Infusion Rate	Infusion Duration
	ml/hr	*μmoles/ml*	*μmoles/ g wet wt*	*μmoles/ g H_2O*		*ml/hr*	*min*
Group I, no infusion							
P-1	0.52	74	58	72	0.97		
P-2	0.50	76	70	84	1.11		
P-3	0.12	48	68	84	1.75		
P-5	0.60	45	83	104	2.34		
P-6	0.45	39	71	88	2.25		
P-7	0.50	133	151	189	1.42		
P-9	0.06	323	399	499	1.55		
P-10	0.12	85	102	128	1.50		
				Average	1.61		
Group II, 15% mannitol infusion							
P-13	0.12	43	107	134	3.10	0.25	30
P-14	0.14	44	95	119	2.71	0.25	30
P-15	0.18	104	140	174	1.68	0.25	30
P-16	0.12	8	86	107	13.2 †	0.25	30
P-17	0.16	60	65	81	1.36	0.25	30
P-18	0.40	33	64	80	2.47	0.25	30
P-19	2.8	28	47	59	2.08	4.8	100
P-20	2.6	21	47	58	2.80	4.8	100
				Average	2.31		

* Urine collected from catheter in ureter; flow from one kidney.

† Because of the aberrant value for urine urea concentration, data from this rat have been omitted from calculation of the averages.

In order to make certain that the urea concentrations found in papilla and medulla represented those present at the time that the urine was formed, urine was collected directly from the catheterized ureter of anesthetized (ether) rats. This procedure also served to exclude significant losses of urea during flow through ureter and bladder. When relative stability of urine flow and urea con-

TABLE III

Urea concentration in the urine and papilla of rats maintained on a chow diet and infused with 15% mannitol

Rat	Urine* flow	Urine urea	Papillary urea	Papillary urea	Papilla / Urine	Infusion Rate	Infusion Duration
	ml/hr	*μmoles/ml*	*μmoles/ g wet wt*	*μmoles/ g H_2O*		*ml/hr*	*min*
S-1	0.08	939	454	545	0.58	0.25	30
S-3	0.12	1,252	493	617	0.49	0.25	30
S-4	0.06	908	557	696	0.77	0.25	30
S-5	0.60	167	149	187	1.12	0.40	150
S-6	0.30	481	363	454	0.94	0.40	180
S-7	0.18	904	496	620	0.69	0.40	150
S-8	0.10	1,004	515	644	0.64	0.40	135
S-9	0.34	307	170	212	0.69	0.86	120
S-10	0.94	155	138	172	1.11	2.8	90
S-11	2.2	70	61	76	1.09	2.8	105
S-12	2.4	48	60	75	1.58	4.8	90
S-13	2.8	38	58	73	1.90	4.8	180
S-14	3.2	39	51	64	1.64	4.8	180
S-15	2.4	52	55	68	1.32	4.8	195
S-16	3.4	45	49†	61	1.34	4.8	210

* Flow from one kidney. Urine collected from catheterized ureter in ether-anesthetized animal.

† Value used is urea concentration in inner medulla. Papillary urea concentration = 36.0 μmoles per g wet weight.

centration had been established by successive urine collections, the animals were sacrificed and tissue analyses carried out as described above.

The results of experiments in rats maintained on a low protein, high salt diet are presented in Table II. Group 1 (Rats P-1 to P-10) received no infusion prior to collection of the urine. In all but one of the rats in this group, the urea concentration in the papilla was higher than that in the urine, with an average concentration ratio of 1.61. These findings establish more conclusively that, in the rat kidney, urea may be accumulated at a higher concentration in the medullary tissue than in the urine. Rats in group 2 were studied at two rates of mannitol infusion. In all rats in this group the tissue urea concentration was higher than the urea concentration in the urine, with an average papilla to urine ratio of 2.31 and a range of 1.36 to 3.10 (excluding a single aberrant value of 13.2).

The ability of the kidneys of rats on a low protein diet to maintain a significant papilla to urine gradient for urea during mannitol diuresis led to a series of experiments in which mannitol was infused into normal rats to test whether the low protein diet was essential to this phenomenon or whether a reduced urea concentration of the urine was itself the determining factor. Sixteen rats that had been fed a chow diet were anesthetized with ether, and one ureter was catheterized. They were infused with 15 per cent mannitol at rates varying from 0.25 to 4.8 ml per hour for periods varying from 30 to 210 minutes before urine was collected and the animals sacrificed for the performance of tissue analyses. The results are presented in Table III. As the rate of mannitol infusion was increased, the urinary urea concentration fell and, with the fall in urine urea concentration, the papillary urea concentration became equal to or higher than the urinary urea concentrations. In Rats S-5 and S-10 to S-16 in which the urea concentration of the papilla exceeded that in the urine, the urea concentration in the urine was less than 170 μmoles per ml.

DISCUSSION

The possibility that urea might be actively transported in the mammalian kidney has been discussed in detail by Schmidt-Nielsen (16) but direct evidence has been limited to the work in sheep. Studies of kidney slices from sheep which have been maintained on a low protein diet (1.9 per cent) show a higher urea concentration in the medulla than in the urine (25). The highest urea concentration in kidney slices from these sheep was regularly found in the inner zone of the outer medulla. The urea concentration in the inner medulla was always lower than in the outer medulla but still higher than in the urine. Schmidt-Nielsen and O'Dell (25) have suggested that urea might be actively transported by the thick portion of the ascending limb of Henle's loop and that the collecting duct of the sheep might be impermeable to urea. The findings in the rat differ from those of Schmidt-Nielsen and O'Dell in the sheep in several respects: in the rat, the highest tissue urea concentrations were found in the papilla or inner medulla, and the concentrations in both of these regions were higher than in the inner stripe of the outer medulla; and the urea concentration in the inner stripe of the outer medulla was usually lower than that in the urine.

There are two mechanisms by which the urea concentration in the medullary tissue might become higher than the urine urea concentration. The first of these involves transport of urea by the loop of Henle. The work of Wirz, Hargitay and Kuhn (26–28), Gottschalk and Mylle (29), and Berliner and co-workers (12) has focused attention on the importance of the countercurrent multiplier of the loop of Henle in producing hypertonicity in the medulla and consequently in the urine. The over-all effect of such a process is to deposit solute without water in the renal medulla by the active transport of sodium out of the loop of Henle and to remove the excess water as hypotonic fluid in the loop of Henle (27, 29, 30). Under most circumstances sodium is believed to be the solute which is actively transported in the loop. It is conceivable that urea could be actively transported by the loop of Henle and its concentration multiplied by the countercurrent flow in this structure.

The micropuncture studies of Lassiter, Gottschalk and Mylle (31) appear to eliminate the loop of Henle as the site of active urea transport. They have shown that the tubular fluid to plasma ratio for urea in the proximal tubule is 1.5 and for inulin 3.0. In the first portion of the distal tu-

bule, this ratio for urea is 7.7 and for inulin 6.9. Thus, in the normal rat kidney more urea leaves the medulla in the loop of Henle than enters from the proximal tubule, and this excludes a countercurrent multiplication of urea concentration by the loop as primarily responsible for the accumulation of urea under the conditons of their study.

The other structure that might transport urea is the collecting duct. Most of the evidence on the role of urea in the medullary concentrating mechanism in the dog implies that during antidiuresis, the collecting duct is permeable to urea, and that urea accumulates along a concentration gradient created by the removal of water from the collecting duct. Jaenike (13) has shown that, in the dog under the influence of vasopressin, the permeability of the collecting duct to urea is increased. However, in the experiments reported here the collecting duct of the rat on a low protein diet appears to be relatively impermeable to urea. In normal rats on a chow diet the urine urea concentration is invariably higher than the urea concentration in the papilla. Under these circumstances the medullary urea might rise by passive diffusion from the collecting duct. However, the infusion of 15 per cent mannitol into normal rats will lower the urea concentration in the urine and bring out the ability of the papilla to concentrate urea. Unless the permeability of the collecting duct changes during the infusion of mannitol, it would be reasonable to assume that the collecting duct of the rat is impermeable to urea on both normal and low protein diets. Thus, under most conditions, urea appears to enter the medulla by a carrier-mediated process of facilitated diffusion. Only by lowering the urea concentration in the collecting duct sufficiently would it be possible to bring out the ability of the rat kidney to concentrate urea.

Transport of urea across the collecting duct from urine to peritubular space would operate in two ways to concentrate the urine: *1*) by reducing the volume of fluid remaining in the collecting duct, and *2*) by raising the medullary urea concentration above that in the collecting duct. When the concentration of urea in the collecting duct is higher than that in the peritubular space, the effect of urea transport would be identical with that which could be achieved by the passive diffusion of urea, as in the mechanism proposed by Berliner and associates (12) and Jaenike (13). When the concentration of urea in the collecting duct is equal to that in the medullary interstitium, active transport of urea would raise the interstitial urea concentration at the expense of that in the urine. This would increase the concentration of non-urea solute in the urine in accord with the observation of Crawford and co-workers (21) but would not yield a further increase in total urine concentration. Furthermore, the reduction in urine volume resulting from urea transport would make possible a greater increase in urine concentration from a given amount of sodium transported by the loops.

The urea gradient that can be achieved between medullary interstitium and collecting duct fluid should depend upon a number of factors: *1*) the rate of transport of urea from tubular fluid to peritubular space; *2*) the rate of diffusion of urea from medulla back into the collecting duct; *3*) the rate at which urea is lost from the medulla by the countercurrent flow in the blood vessels and in the loop of Henle; and *4*) the proportion of non-urea solute in the urine. A high permeability of the collecting duct to water and a relative impermeability to urea assumed, the ability to demonstrate urea accumulation by the medulla will depend upon the rate at which urea is removed from the collecting duct, and the proportion of non-urea solute in the collecting duct. In the experiments with rats on a low protein diet where sodium chloride accounted for most of the solute in the collecting duct, or in the experiments with mannitol infusions in which mannitol was the principal solute, a low rate of urea transport by the collecting duct would have become apparent as a higher concentration of urea in the medulla than in the urine. In experiments where urea comprised a larger proportion of the solute in the collecting duct, a gradient for urea could not be demonstrated between medulla and urine, presumably because the rate of urea transport by the collecting duct was too low relative to the amount of urea in the collecting duct.

Klümper, Ullrich and Hilger (32) have demonstrated that urea is lost from the collecting duct of the golden hamster during antidiuresis. Although consistent with passive movement of urea

from collecting duct to interstitium, the observations reported here on the rat, and those of Lassiter and associates (31), introduce the possibility that the movement of urea out of the collecting duct of the hamster is due to active transport.

All micropuncture studies in mammals have used rodents as the experimental animal, but the data so obtained have been applied to other mammalian species under the assumption that the kidneys of all species behave in a similar way. The observations presented here which support the concept that urea is actively transported by the collecting duct of the rat differ from observations under similar conditions in the dog in which urea is thought to accumulate in the inner medulla by passive diffusion from the collecting duct. Such findings re-emphasize the need for caution in generalizing, to dogs or other species, micropuncture data obtained from rodents.

SUMMARY

Rats on a low protein, high salt diet receiving supplementary urea produced urine containing a significantly higher non-urea solute concentration than did rats on a similar diet but without urea supplements. The concentration of urea in the papilla and inner medulla from these and other rats on a low protein, high salt diet was as much as three times the concentration of urea in 24-hour urine collections. Experiments with normal and protein-depleted rats, in which urine was collected from a single ureter under ether anesthesia, further showed that the demonstration of urea accumulation in the medulla of the rat appears to be dependent upon the presence of a low urinary urea concentration and, hence, a considerable proportion of non-urea solute. The results of these experiments indicate that: *1*) urea supplements to a low protein, high salt diet increase the concentration of non-urea solute in rat urine; *2*) the rat kidney is able to concentrate urea in the medulla, presumably by an active transport mechanism located in the collecting duct; and *3*) under the conditions of these experiments the collecting duct of the rat must be relatively impermeable to urea.

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CORRECTION

On page 1287 of the article entitled "Glucagon Antibodies and an Immunoassay for Glucagon" by Roger H. Unger, Anna M. Eisentraut, M. S. McCall and Leonard L. Madison (J. clin. Invest. 1961, **40**, 1280), the units $\mu\mu$equivalents and μEq are incorrect in Table III and in the text below. In the table, the unit of measurement for B/F ratios and glucagon concentrations should be $\mu\mu$g equivalents per ml and the total glucagon content of organ at the bottom of column 1 should be in μg equivalents. In the text, lines 7–12 should read: "The estimated glucagon concentrations are expressed in micromicrogram equivalents of beef-pork glucagon per milliliter. Calculations of the total glucagon content of pancreas of both species range from 12.5 to 36.3 μg Eq of beef-pork glucagon."

A STUDY OF THE HYPERURICEMIA INDUCED BY HYDROCHLOROTHIAZIDE AND ACETAZOLAMIDE SEPARATELY AND IN COMBINATION *

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The mechanism by which the chlorothiazide drugs produce hyperuricemia is not known, but the frequent and simultaneous reduction in renal clearance of uric acid suggests a tubular effect (1–3). Gutman, Yü and Sirota (4) have demonstrated a similar decrease in uric acid clearance after acetazolamide [1] therapy, and more extensive studies by Frascarelli, Lucidi and Cozzali (3) have not only confirmed this but have shown that levels of hyperuricemia induced by acetazolamide equal those produced by chlorothiazide [2] and hydrochlorothiazide.[3] The present studies were designed to confirm these previously reported findings, and to determine whether the effects of these drugs were additive. Complete urinary uric acid excretions and clearances were also done to test the dependence of the hyperuricemia upon diminished output.

The findings confirm those of Frascarelli and associates but differ in indicating a greater hyperuricemic activity for hydrochlorothiazide than for acetazolamide, and in demonstrating an additive effect when these drugs are used in combination. Still more significantly, these studies show that in low doses, hydrochlorothiazide increased the excretion of uric acid with an associated hyperuricemia.

METHODS

Normal, healthy medical students were used as the subjects for this study. They were required to ingest low purine diets, starting 48 hours prior to the first day of study, and their use of salicylates, vitamins and caffeine-containing beverages was curtailed. Fasting blood specimens were collected each morning at intervals of 24 hours; the plasma was separated and frozen until time for chemical analysis. For the duration of the study each subject collected his total output of urine in 24-hour aliquots, using 1-gallon plastic containers with toluene as a preservative. These specimens were analyzed daily, immediately after measurement of each total volume, except at weekends when the urine aliquots were diluted 1:10 with distilled water and stored at 4° C. The purpose of these dilutions was to prevent precipitation of uric acid with its loss to chemical quantitation.

Uric acid was measured according to Brown's modification of the urea cyanide colorimetric method, and creatinine content was determined by Phillip's modification of the alkaline picrate method (5). Duplicate analyses were performed on all specimens, and when the creatinine content varied by more than 10 per cent from the mean (or if the subject reported inadvertent or accidental loss of significant degree), the specimen was eliminated from the study. As a precaution against the possibility that a drug chromagen might interfere with these colorimetric analyses, the uricase method of Praetorius and Poulsen (6) was used to check samples of blood and urine.

RESULTS

Plasma studies. The following data refer only to *a*) the plasma uric acid levels just prior to and following medication, and *b*) the group of subjects with control uric acid levels falling below the usually accepted upper normal limit of 6.0 mg per 100 ml. The initial (control) specimen was taken after at least 48 hours of low purine diet and immediately preceding the administration of drug. The next three plasma uric acid values refer to specimens drawn 24, 48 and 72 hours after the control, during which intervals all subjects except those in the control group continued the medication. Table I lists the mean uric acid levels (calculated to two decimal places) and the range of the daily levels found in each of the four groups of subjects studied.

The control group consisted of 15 subjects

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[1] Diamox, Lederle (2-acetylamino-1,3,4-thiadiazole-5-sulfonamide).

[2] Diuril, Merck (6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide).

[3] Hydrodiuril, Merck (6-chloro-7-sulfamyl-3,4 dihydro-1,2,4 benzothiadiazine-1,1-dioxide).

TABLE I

Plasma uric acid levels: mean values and range *

Group, No.	Time in hours 0	24	48	72	Difference between 0–72 hours	Significance of difference
Control, 15	4.94 3.4–5.8	4.99 3.6–5.8	4.85 3.7–5.8	4.86 3.5–5.5	−0.08 −0.6 to +0.4	>0.05
Acetazolamide, 14	4.72 3.5–5.6	5.07 3.7–6.2	5.39 4.1–7.2	5.31 4.0–6.1	+0.59 −0.1 to +1.5	<0.01
Hydrochloro., 12	4.62 2.7–5.9	4.97 3.3–6.0	5.40 3.7–6.6	5.49 4.2–6.8	+0.87 +0.2 to +1.6	<0.01
Acet. and hydro., 11	4.77 3.4–5.4	5.37 3.3–6.4	5.99 4.2–7.1	6.53 4.8–8.1	+1.76 +0.8 to +2.8	<0.01

* Values are in mg per 100 ml plasma. All subjects were maintained on a low purine diet.

restricted to low purine diets but given no drug. These levels ranged from 3.4 to 5.8 mg per 100 ml (mean, 4.94) on the first day, and 3.5 to 5.5 mg per 100 ml (mean, 4.86) on the third and final day. Although the individual daily variations ranged up to 1.0 mg per 100 ml, these occurred at random without a discernible trend or pattern for the group.

In the second group were 14 subjects who received acetazolamide in doses of 1 g (250 mg at 9 a.m., 1 p.m., 5 p.m. and 9 p.m.) each day for 3 days. Their initial plasma uric acid levels ranged from 3.5 to 5.6 mg per 100 ml (mean, 4.72) and their levels rose after medication. The magnitude of these variations was only slightly greater than that of the control group, but a clear and consistent trend was apparent, with the plasma uric acid rising to levels of 4.0 to 6.1 mg per 100 ml (mean, 5.31) by the third day. Only 1 individual in this group of 14 failed to increase his third-day level over his control level.

Each of the 12 subjects in the third group received 50 mg hydrochlorothiazide (25 mg at 9 a.m. and 5 p.m.) daily for 3 days. Initial plasma uric acid values in this group ranged between 2.7 and 5.9 mg per 100 ml (mean, 4.62) and after 3 days of drug ingestion, all 12 rose to new levels varying from 4.2 to 6.8 mg per 100 ml (mean, 5.59).

Each of the 11 subjects in the fourth group received 50 mg hydrochlorothiazide and 500 mg acetazolamide (in divided doses at 9 a.m. and 5 p.m.) daily for 3 days. In this group the initial plasma uric acid values ranged from 3.4 to 5.4 mg per 100 ml (mean 4.77). After therapy the levels increased in all 11 subjects; the third and final readings extended from 4.8 to 8.1 mg per 100 ml (mean, 6.53).

In each of these four groups the uricase method of Praetorius and Poulsen was used to check the results of three of the subjects and to confirm the relative changes in plasma uric acid levels.

Statistical analysis of each of these groups (comparing the initial with the final plasma levels) by Wilcoxan's signed-rank test (7) shows that variations in plasma uric acid levels were insignificant in the control group, but significant with a probability of less than 0.01 for those medicated with acetazolamide and hydrochlorothiazide separately and in combination.

By using the Wilcoxan-White two-sample rank test (7) to compare each of these groups with the

TABLE II

*Uric acid renal excretion and clearance studies while receiving acetazolamide (Subject R.H.)**

Day	Plasma uric acid	Urine uric acid	Mean urine uric acid	Uric acid clearance
	mg%	*mg/24 hrs*	*mg/24 hrs*	*ml/min*
C 1	4.2	723		12.0
C 2	4.3	711		11.5
C 3	4.6	708		10.7
C 4	4.7	754	724	11.1
Rx 5	5.2	583		7.8
Rx 6	5.9	490		5.8
Rx 7	5.7	612		7.5
Rx 8	5.9	594		7.0
Rx 9	5.5	656		8.3
Rx 10	5.1			
Rx 11	5.4	697	605	9.0
PRx 12	5.4	578		7.4
PRx 13	4.4	710		11.2
PRx 14	4.2	634		10.5
PRx 15	4.4	838	690	13.2

* C: control period, low purine diet; Rx: drug period (acetazolamide, 1 g in 4 divided doses daily for 7 days); PRx: post-drug period with low purine diet continued.

TABLE III

*Uric acid renal excretion and clearance studies while receiving acetazolamide *: mean values and ranges*

Subject		Control period 4 days		Drug period 4 days		Post-drug period 4 days	
		Mean	Range	Mean	Range	Mean	Range
S.A.	Plasma uric acid†	3.6	3.5–3.7	4.1	4.0–4.2	3.8	3.3–4.1
	Urine uric acid‡	678	585–743	575	527–635	675	610–738
	Clearance§	13.1	11.3–14.0	9.8	8.7–10.8	12.6	10.3–15.5
L.R.	Plasma uric acid	3.9	3.8–3.9	4.4	4.1–4.7	3.5	3.4–3.7
	Urine uric acid	496	434–550	433	402–478	503	450–562
	Clearance	9.1	7.7–10.5	6.9	5.9–8.1	9.9	9.2–10.5
M.D.	Plasma uric acid	6.5	6.2–6.8	7.2	7.0–7.6	6.6	5.9–7.0
	Urine uric acid	622	602–632	532	458–641	559	518–635
	Clearance	6.6	6.5–6.7	5.1	4.5–6.4	6.0	5.2–7.5

* Acetazolamide, 1 g daily in 4 divided doses.
† Mg/100 ml plasma.
‡ Mg/24 hrs.
§ Ml/min.

others, no significant variation is found in the initial plasma levels of all four groups. However, the elevated third-day plasma levels in the acetazolamide, hydrochlorothiazide and combined-drug groups differ significantly from the corresponding final levels in those receiving no drug, with p values of less than 0.01. Comparison of the degree of change in plasma uric acid levels induced by acetazolamide and hydrochlorothiazide shows the increased levels in the hydrochlorothiazide group to be significant, with p equal to 0.05. Moreover, the hyperuricemia produced by combined therapy is significantly greater than that of each drug alone, with p less than 0.01.

Plasma and urine studies. The hyperuricemia induced by acetazolamide is associated with a decreased urinary excretion of uric acid. Table II lists the data obtained from Subject R.H. During the 4-day control period his calculated renal clearance of uric acid ranged from 10.7 to 12.0 ml per minute. Acetazolamide caused a relative hyperuricemia and a diminished excretion of uric acid, with a marked decrease in the renal clearance (which fell to levels ranging from 5.8 to 9.0 ml per minute). In the recovery period after the drug was discontinued, no increased excretion of uric acid was found, as one would expect if the hyperuricemia were purely the result of renal retention.

TABLE IV

Uric acid renal excretion and clearance studies while receiving hydrochlorothiazide (Subject M.G.) and hydrochlorothiazide and acetazolamide (Subject B.B.†)*

Day‡	M.G.				B.B.			
	Plasma uric acid	Urine uric acid	Mean urine uric acid	Uric acid clearance	Plasma uric acid	Urine uric acid	Mean urine uric acid	Uric acid clearance
	mg%	*mg/24 hrs*	*mg/24 hrs*	*ml/min*	*mg%*	*mg/24 hrs*	*mg/24 hrs*	*ml/min*
C 1	4.9	682		9.7	5.5	675		8.5
C 2	4.7	600		8.9	5.4	808		10.4
C 3	4.9	622		8.8	5.1	658		9.0
C 4	5.2	799	676	10.7	5.2	686	707	9.2
Rx 5	6.0	576		6.7	5.9	509		6.0
Rx 6	7.2	537		5.2	7.1	473		4.6
Rx 7	7.6	562		5.1	8.0	596		5.2
Rx 8	8.3	782	614	6.5	8.1	640	555	5.5
PRx 9	7.4	721		6.8	7.5	754		7.0
PRx 10	6.8	891		9.1	6.6	905		9.5
PRx 11	6.6	673		7.1	5.6	849		10.5
PRx 12	6.4	736	755	8.0	4.7	1,025	883	15.1

* Hydrochlorothiazide, 200 mg daily in 4 divided doses for 4 days.
† Hydrochlorothiazide (50 mg) and acetazolamide (500 mg) daily in 2 divided doses for 4 days.
‡ As in Table II.

TABLE V

*Uric acid renal excretion and clearance studies of a single subject (C.R.) receiving hydrochlorothiazide alone * (A) and later in combination with acetazolamide † (B)*

Day‡	A				B			
	Plasma uric acid	Urine uric acid	Mean urine uric acid	Uric acid clearance	Plasma uric acid	Urine uric acid	Mean urine uric acid	Uric acid clearance
	mg%	*mg/24 hrs*	*mg/24 hrs*	*ml/min*	*mg%*	*mg/24 hrs*	*mg/24 hrs*	*ml/min*
C 1	3.2	530		11.5				
C 2	3.0	396		9.2	3.7	542		10.2
C 3	3.5	543		10.8	3.7	446		8.4
C 4	3.8	540		9.9	3.4	583		11.9
C 5	3.5	504	503	10.0	3.4	506	519	10.3
Rx 6	4.1	520		8.8	3.3	553		11.6
Rx 7	4.1	598		10.1	4.2	383		6.3
Rx 8	4.2	570		9.4	4.8	518		7.5
Rx 9	5.2	586		7.8	5.6	609		7.6
Rx 10	4.5	527		8.1	5.8	558		6.7
Rx 11	5.1	598		8.1	5.2	657		8.8
Rx 12	4.7	691		10.2	5.5	512		6.5
Rx 13	4.8	652	593	9.4	5.0	656	556	9.1

* Hydrochlorothiazide, 50 mg daily in 2 divided doses for 8 days.
† As above, and acetazolamide 500 mg daily in 2 divided doses for 8 days.
‡ As in Table II.

These results are comparable with those found in three additional subjects who received the same dose of acetazolamide for 4 days each. Their data are summarized in Table III.

The potentiation of hydrochlorothiazide hyperuricemia by acetazolamide is also apparent in the data of Table IV. Subjects M.G. and B.B. were matched in age and size; M.G. received 200 mg hydrochlorothiazide daily while B.B. received 50 mg hydrochlorothiazide and 500 mg acetazolamide daily. Despite the difference in the dosage of hydrochlorothiazide, both showed similar qualitative and quantitative responses, with hyperuricemia and decreased urinary uric acid excretion and clearance. After drugs were discontinued, more complete recovery was seen in B.B. Unlike the four subjects who received acetazolamide alone, during their recovery period these two subjects increased their excretions of uric acid considerably above control levels.

TABLE VI

Uric acid renal excretion and clearance studies while receiving hydrochlorothiazide (Subjects W.Z. and J.M.†)*

Day‡	W.Z.				J.M.			
	Plasma uric acid	Urine uric acid	Mean urine uric acid	Uric acid clearance	Plasma uric acid	Urine uric acid	Mean urine uric acid	Uric acid clearance
	mg%	*mg/24 hrs*	*mg/24 hrs*	*ml/min*	*mg%*	*mg/24 hrs*	*mg/24 hrs*	*ml/min*
C 1	4.1	444		7.5	2.8	404		10.0
C 2	4.0	449		7.8	2.8	391		9.7
C 3	4.2	475		7.9	2.5	425		11.8
C 4	4.0	482	463	8.4	2.7	408	407	10.5
Rx 5	4.4	451		7.1	3.3	482		10.2
Rx 6	5.4	504		6.5	3.7	559		10.5
Rx 7	4.4	439		7.0	4.2	517		8.5
Rx 8	5.1	603		8.2	4.8	565		8.2
Rx 9	4.9	590		8.4	5.1	539		7.3
Rx 10	4.9	576	527	8.2	5.2	774		10.3
Rx 11					4.6	693	590	10.5
PRx 12	4.8	672		9.7	3.8	688		12.6
PRx 13	4.6	675		10.2	3.3	594		12.5
PRx 14	3.5	705		14.0	2.8	543		13.4
PRx 15	3.4	694	687	14.2	2.8	643	617	16.0

* Hydrochlorothiazide, 75 mg daily in 3 divided doses for 6 days.
† Hydrochlorothiazide, 50 mg daily in 2 divided doses for 7 days.
‡ As in Table II.

A similar study was done on a single subject; C.R. initially received 50 mg hydrochlorothiazide daily for 8 days, and 4 weeks later, 50 mg hydrochlorothiazide and 500 mg acetazolamide daily for 8 days (Table V). Combined therapy produced the greater degree of hyperuricemia and the greater fall in the calculated renal clearance of uric acid but, during each procedure, the uric acid excretion under medication exceeded the control. The mean control uric acid excretions were 503 and 519 mg per 24 hours, while the mean excretions during drug therapy were 593 and 556 mg per 24 hours.

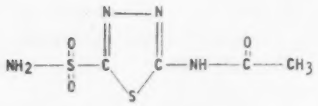
These results were confirmed in four addtional subjects, two of whom are reported here. W.Z. received 75 mg hydrochlorothiazide daily for 6 days, and J.M. received 50 mg hydrochlorothiazide daily for 7 days (Table VI). In both cases the urinary excretions and plasma levels of uric acid rose, while the calculated renal clearances diminished slightly but returned to control levels before drugs were discontinued. Moreover, and still more significantly, this hyperuricosuria persisted even after medication was discontinued, and in both instances the renal clearance of uric acid rose to levels exceeding those of the initial control periods. The plasma and urine of two of these subjects were analyzed by the uricase method as well and the results confirmed.

DISCUSSION

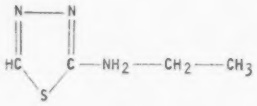
Our data support previous observations (3, 4) that acetazolamide can induce hyperuricemia and decrease the renal excretion and clearance of uric acid. Confirmatory analysis of plasma and urine by the uricase method establishes that these changes and measurements truly represent uric acid. Also confirming this activity of acetazolamide is its ability to potentiate the hyperuricemia induced by hydrochlorothiazide.

These data support the concept that this effect of acetazolamide is renally mediated. However, the observation that small doses of hydrochlorothiazide produce an increased excretion of uric acid is not compatible with the concept of renal retention as the mechanism for the associated hyperuricemia. The dual properties of the chlorothiazide drugs in increasing as well as decreasing the excretion of uric acid have previously been noted and likened to the paradoxical actions of uricosuric agents such as acetylsalicylic acid and probenecid (2, 8). Yü and Gutman feel that these drugs may exert either of two effects on the renal excretion of uric acid (9): at low concentrations they supposedly reduce the tubular secretion and consequently the excretion and clearance of uric acid; at higher dosage they inhibit the tubular reabsorption and increase the excretion and clearance. However, our data show that the increased excretion of uric acid occurs at lower doses of hydrochlorothiazide and is accompanied by hyperuricemia and a slight decrease or no change in renal clearance. This pattern of events could be produced by either mobilization of urate from the miscible pool or a decrease in the nonrenal disposal of uric acid (10), but neither of these mechanisms could account for the further increase in urinary uric acid excretion that occurred immediately after hydrochlorothiazide was discontinued. An alternative explanation is that the drug increased the production of uric acid. Uricogenesis can be produced by drug therapy, as demonstrated by Krakoff and Balis, using ethylaminothiadiazole (EATDA) (11).

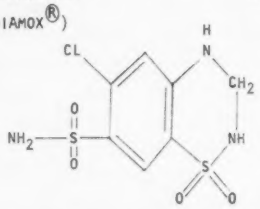
The close chemical relationship of acetazolamide (2-acetylamino-1,3,4 thiadiazole-5-sulfonamide) and EATDA (2-ethylamino-1,3,4 thiadiazole) (see Figure 1) suggests a possible mechanism by which hydrochlorothiazide, in varying doses, can increase or decrease the urinary excretion of uric acid. Krakoff and Balis found that EATDA greatly enhanced the *de novo* synthesis of purine nucleotides and did not increase the yield of uric acid from nucleic acids. Actually, their data indicate a decreased yield of uric acid from nucleic acid. EATDA may have a dual effect on uric acid metabolism, at least one of which is shared with acetazolamide. If EATDA does decrease the degradation of nucleic acids, a loss of feedback inhibition by purine or purine nucleotide salvage mechanisms could result in greatly increased *de novo* synthesis of purine nucleotides (12–14). Acetazolamide could have the same initial effect on nucleic acid degradation but could block the compensatory synthesis of purine nucleotides in a manner similar to the inhibition of bacterial nucleotide synthesis by the sulfonamide drugs (15). Hydrochlorothiazide could act similarly, with low doses allowing, and higher doses



2 - ACETYLAMINO - 1, 3, 4 - THIADIAZOLE - 5 - SULFONAMIDE (ACETAZOLAMIDE, DIAMOX®)



2 - ETHYLAMINO - 1, 3, 4 - THIADIAZOLE (EATDA)



3, 4, DIHYDROCHLOROTHIAZIDE
HYDROCHLOROTHIAZIDE
HYDRODIURIL®

FIG. 1. DRUGS PRODUCING HYPERURICEMIA.

inhibiting, the compensatory *de novo* synthesis of purine nucleotides.

SUMMARY

The data presented support the concept that acetazolamide produces a relative hyperuricemia and a decreased renal excretion and clearance of uric acid. Although this hyperuricemia is not so great as that produced by hydrochlorothiazide, the two drugs together have an additive effect.

Hydrochlorothiazide, in low doses, produces a relative hyperuricemia with increased excretion of uric acid and without marked changes in its renal clearance.

The possibility is discussed that these drugs may exert an effect on uric acid through complex metabolic pathways rather than a renal tubular one.

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DIURNAL CYCLE OF GLOMERULAR FILTRATION RATE AND SODIUM AND CHLORIDE EXCRETION DURING RESPONSES TO ALTERED SALT AND WATER BALANCE IN MAN *

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A diurnal cycle in volume and composition of the urine in man has long been recognized. For the most part, excretion of water and electrolytes is lowest toward the end of the sleeping period and is greatest at some time during the hours of normal wakefulness. Descriptions of the diurnal cycle of sodium chloride excretion have been published by Völker (1), Campbell and Webster (2), Kleitman (3), Simpson (4–6), Norn (7), Manchester (8), Barbour and co-workers (9), Borst and de Vries (10), Gerritzen (11), Stanbury and Thomson (12), Azérad, Lestradet, Reinberg and Gatha (13), Lewis and Lobban (14–16), Payne and de Wardener (17) and Doe, Vennes and Flink (18) and in other studies noted below. The cycle does not depend upon meals, activity or salt content of the diet, since it persists during fasting or evenly spaced feeding (7, 12, 19–21), during complete rest (2, 12, 21) and on sodium restricted or supplemented diets (7, 12). Although the cycle can be disturbed, but not abolished, by sleeplessness (3, 5) and is reversed in night workers (5, 7), the normal 24-hour cycle is retained, at least temporarily, in subjects on 12-hour sleep-activity cycles (22) and during the first 24 to 72 hours after acute reversal of the sleep rhythm (3, 7, 19). The excretory cycle can be partially dissociated from the sleep-activity rhythm in subjects whose daily routine is organized around a 22 or 27 hour day, but the degree of dissociation between the two rhythms seldom exceeds 2 hours (15, 16).

Changes in body fluid content such as may attend variations in salt and water intake are followed by alterations in the mean daily excretion rate of salt and water which, directionally, are such as to minimize the disturbance in body composition. Although these alterations may be described in several ways, they represent, in effect, changes in the level, amplitude or form of the diurnal cycle. The mechanisms that determine the character of the diurnal excretory cycle and alter the cycle after changes in body fluid balance are largely inferential but appear to involve changes both in the rate of extrarenal secretion of humoral agents which modify renal tubular transport of electrolytes and water, and in the rate of glomerular filtration of salt and water. Studies relating to a possible role of filtration rate in these mechanisms have been reviewed recently (23).

Glomerular filtration rate exhibits a diurnal cycle, increasing by day and decreasing by night, which tends to parallel the sodium chloride excretory cycle (12, 24). In subjects with congestive heart failure or cirrhosis, in whom the excretory cycle is frequently reversed, the filtration-rate cycle is generally reversed also (25–27). Correspondingly, filtration rate is generally elevated above control values after chronic loading with saline (28) or after chronic water retention obligated by vasopressin (Pitressin tannate) injections (29, 30) and is depressed in volunteer subjects or patients on low salt diets (31–36). In subjects or patients in whom salt retention is induced by steroid (usually deoxycorticosterone) administration, filtration rate is generally increased after several days of salt accumulation and at the time that control rate of salt excretion has been regained (37–41).

From none of these studies, however, is information available concerning the diurnal cycle of glomerular filtration rate during states of altered

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fluid balance, the changes in this cycle during the transition from one to another state, and correlation (or lack of it) between changing filtration rate and changing sodium chloride excretion during the transition phase. The studies reported here were undertaken to examine these questions.

METHODS

The experiments were performed on 3 healthy male subjects. Because of their familiarity with the techniques and theory involved, all three are considered trained subjects. A dietary history was obtained prior to an experiment. A standard single day's menu, closely approximating the usual diet, was then prepared with the collaboration of the subject. This menu was repeated each day of the study, subject only to modification of the salt or water content. One to two days before beginning the clearances, the subject was admitted to a private hospital room or to a metabolic study unit to become adjusted to the diet and to the routine of restricted activity, regular urine collection and other procedures. When the protocol was functioning smoothly, clearance measurements were begun.

Priming quantities of inulin and *p*-aminohippurate (PAH) were calculated to produce plasma concentrations of 30 and 1.5 mg per 100 ml, respectively. A sustaining solution of 5 per cent inulin and 1.6 per cent PAH was injected intravenously at 0.6 to 0.7 ml per minute by a Sigmamotor pump through an indwelling plastic catheter. This solution, although supersaturated with respect to inulin, will remain clear for weeks if care is taken to avoid seeding with solid inulin. PAH was omitted from some experiments where avoidance of the additional sodium load in the PAH was considered more important than measurement of PAH clearance. When PAH and inulin were infused simultaneously, they were added to distilled water, the PAH contributing sufficient osmotic pressure to prevent hemolysis.[1] When inulin was infused alone, it was added to 5 per cent glucose. In some experiments powdered, refined inulin,[2] refiltered before use (43), was dissolved in the appropriate vehicle. In other experiments, commercial, ampouled inulin (Warner-Chilcott) was employed. Since this inulin is prepared in saline, a quantity sufficient for 24 hours' infusion contains 2.5 g of sodium chloride and an amount of salt approximating this quantity was removed from the patient's diet. The infusion was delivered to the subject through a 10- to 12-foot length of small-bore Tygon tubing which permitted free ambulation within the confines of the study room. Although the subjects were relatively inactive, they were not confined to bed. Clearance periods were generally of 3 hours' duration, but 4-hour periods were employed during sleep in some studies. Inulin clearance was not calculated for periods (see figures) in which large changes in plasma inulin concentration occurred as the result of disturbances of the infusion. Average rate of change in plasma inulin concentration in the remaining periods did not exceed 10 per cent per hour. Urine specimens were routinely collected by voluntary voiding, with the exception of the final clearance period in one experiment (Figure 2) when difficulty in urination required catheterization. Blood specimens were drawn at 3-hour intervals through an indwelling catheter contralateral to the infusion catheter and at either the midpoint or the end of the periods. In some studies, 15-ml specimens were drawn, and no attempt was made to restore the drawn blood. In other studies, in which 25-ml specimens were drawn, the packed cells were suspended in saline and reinjected to minimize possible renal function changes arising from cumulative blood loss. Under such conditions no measurable change in hematocrit could be detected, but approximately 8 g of plasma proteins was removed each day. Weight and temperature were measured frequently; blood pressure and pulse were not measured routinely. Infusions and consecutive clearance periods were continued for 2 to 5 days. Further pertinent methodologic details will be noted during discussion of the individual experiments.

Inulin concentration was measured as follows. To 1 ml each of plasma and urine samples in 25-ml Erlenmeyer flasks was added 8 ml of a 1.0 per cent aqueous suspension of washed yeast. The flasks were lightly stoppered and allowed to stand overnight. One ml each of 10 per cent zinc sulfate and 0.75 N sodium hydroxide (Somogyi precipitation) was added, the flasks were shaken, allowed to stand a few minutes, and the centrifuged supernatant collected by decantation. Inulin in the plasma filtrates and appropriately diluted urine filtrates was measured colorimetrically by the method of Roe, Epstein and Goldstein (44) as modified by Schreiner (45). The above method of yeasting regularly removed 95 per cent of a 200 mg per 100 ml glucose or fructose solution. A plasma blank was obtained by similarly treating 1 ml of inulin-free plasma. Thus measured, the plasma blank is equivalent to about 2 mg per 100 ml plasma inulin and is extremely constant, exhibiting no discernible variation pre- and postprandially or from patient to patient. Most of the color of the blank arises from the yeast. The urine blank, although of nearly the same absolute magnitude as the plasma blank, is so small as a fraction of total urine inulin that it is generally ignored. Yeast blanks and yeast "hematocrits" were not measured. All specimens were routinely analyzed in duplicate *de novo*, and on different days. Determinations on each of the two separate analyses were done in triplicate. A third analysis was performed if the U/P ratios of the first two analyses failed to agree within 5 per cent. Chloride was measured by the method of Van Slyke and Hiller (46), and sodium by flame photometry. PAH clearances and plasma concentrations, and excretion rates

[1] PAH and glucose, when together in solution, form a compound which yields anomalous clearance values (42).

[2] Kindly supplied by the General Diagnostics Division, Warner-Chilcott Corp.

of potassium, calcium, magnesium, and phosphate will be reported in subsequent papers.

RESULTS

Normal diurnal cycle. Diurnal cycles of filtration rate, and plasma concentrations and urinary excretion of sodium and chloride in inactive, healthy subjects on routine diets are shown in the control days of Figures 1–5. The average diurnal cycle in Subject R.S., calculated by averaging for each portion of the cycle the values observed in 5 control days, is shown in Figure 6. The contour of the excretion curves is similar to those reported in other studies. A regular observation in this subject was a brief but marked decrease in excretion coincident with the evening meal and possibly arising from gastrointestinal secretion. Plasma sodium was generally lower during the day but the difference between day and night was small. Chloride concentration varied more widely than did sodium in this subject, usually exhibiting decreases coincident with the morning and evening meals and also possibly related to gastrointestinal secretion. The amplitude of the filtration rate cycle is approximately 20 per cent of the 24-hour mean value in the average cycle. The average amplitude of the separate cycles, however, is 26.5 per cent, and the true amplitude, discoverable by progressively shortening the clearance periods, may well exceed 30 per cent of the 24-hour mean rate.

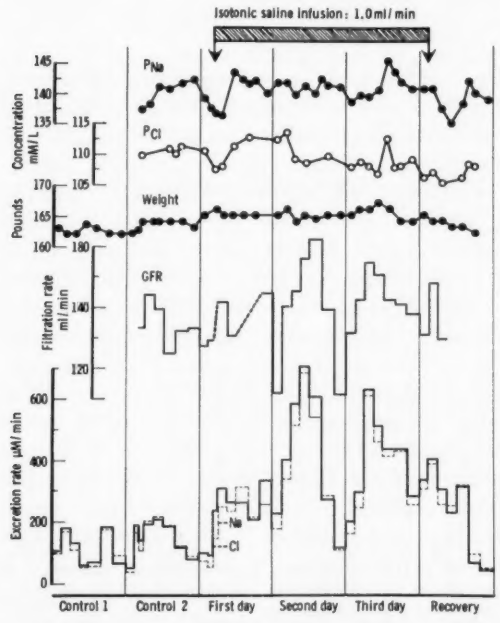

FIG. 1. DIURNAL CYCLE OF FILTRATION RATE AND SODIUM AND CHLORIDE EXCRETION BEFORE AND DURING ISOTONIC SALINE INFUSION. Subject K.B.: age 23; wt 74 kg; ht 173 cm. In this and the following figures, a "day" is 7 a.m. to 7 a.m.

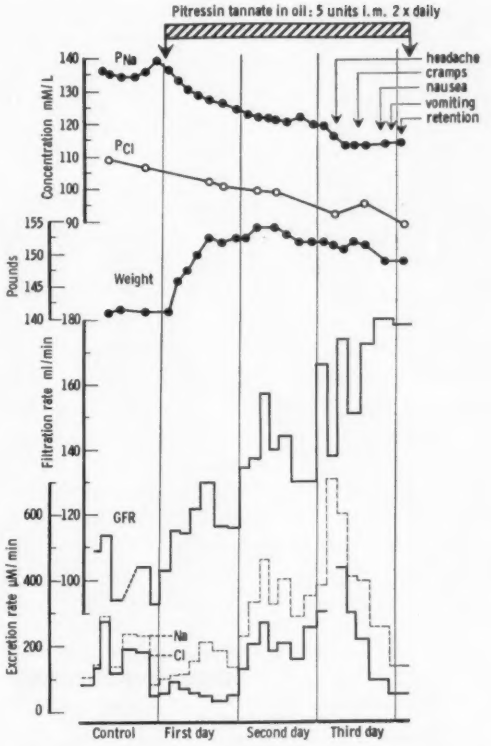

FIG. 2. DIURNAL CYCLE OF FILTRATION RATE AND SODIUM AND CHLORIDE EXCRETION BEFORE AND DURING CHRONIC WATER LOADING SUSTAINED BY VASOPRESSIN INJECTIONS. Para-aminohippurate was infused throughout. Subject R.S.: age 29; wt 65 kg; ht 174 cm.

Diurnal cycle during saline infusion. The diurnal cycles of NaCl excretion and glomerular filtration before and during infusion of isotonic saline are shown in Figure 1. The continuous infusion provided additional salt intake of about 13 g per 24 hours. During the first infusion day, salt excretion increased moderately, the increase being accomplished for the most part by suppression of the normal nocturnal decrease in excretion rate.

Interruptions of the inulin infusion prevented measurement of clearance during several periods, but values in the remaining periods of the first day suggest that daytime inulin clearance did not increase. An increase in plasma chloride concentration at this time may have contributed to the increase in excretion. During the second and third infusion days, diurnal cycles of both excretion and filtration were resumed. As compared with the control, the excretory cycle showed an increase both in minimum (base line) and in the amplitude, together with a narrowing of the nocturnal depression. The filtration rate cycle showed an increase both in amplitude and in the 24-hour mean, the latter rising to 135 and 145 ml per minute on the second and third days, compared with a control value of 128 ml per minute (Table I). The values attained during the brief nocturnal minima, however, were not significantly different (two cycles) from that in the control.

Diurnal cycle during chronic water loading. The diurnal cycles of NaCl excretion and glomerular filtration before and during chronic water loading are shown in Figure 2. On the morning of the first experimental day the subject received the first of repeated injections of vasopressin (Pitressin tannate in oil) and drank 4 L of water over the next several hours. The approximately 10-pound weight increase was thereafter maintained nearly constant by appropriate adjustment of fluid intake.

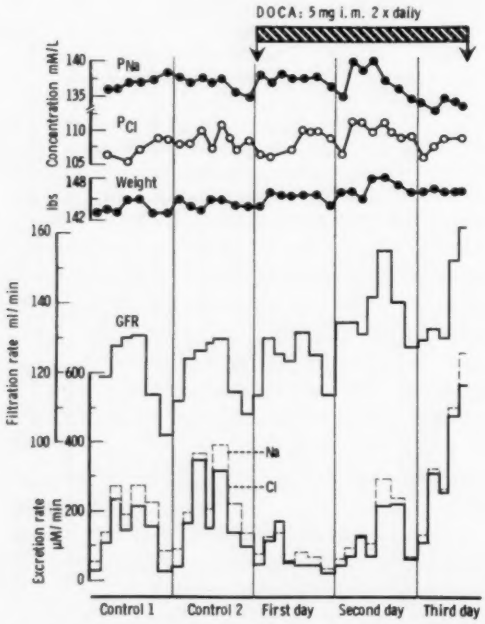

FIG. 3. DIURNAL CYCLE OF FILTRATION RATE AND SODIUM AND CHLORIDE EXCRETION BEFORE AND DURING SODIUM CHLORIDE RETENTION INDUCED BY DEOXYCORTICOSTERONE ACETATE (DCA) INJECTIONS. "Steroid escape" was attained on the third day. PAH was infused throughout; Subject R.S.

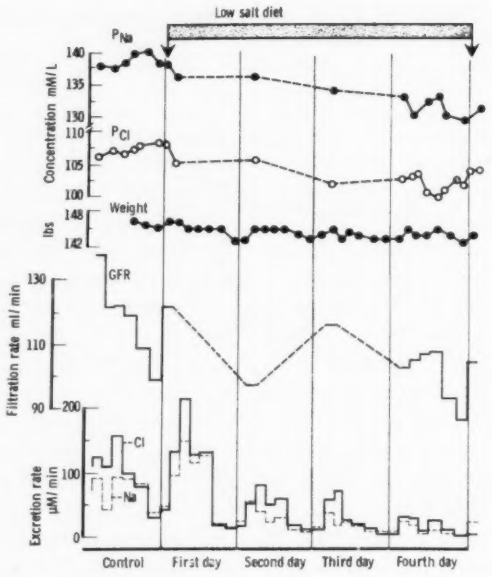

FIG. 4. DIURNAL CYCLE OF FILTRATION RATE AND SODIUM AND CHLORIDE EXCRETION BEFORE AND ON THE FOURTH DAY OF A LOW SALT DIET. Filtration rate was measured briefly each morning of the first 3 days on the low salt diet. PAH was not infused; Subject R.S.

During the first day mean filtration rate was increased (117, compared with 102 ml per minute during the control day). Chloride excretion decreased markedly, while sodium was little changed. Much, if not most, of the decrease in Cl excretion seems attributable, provisionally, to the decrease in plasma Cl concentration. Although some of the excess of sodium over chloride is attributable to *p*-aminohippurate, it seems more likely that a considerable fraction of the sodium excretion is obligated by bicarbonate, as has been reported by Wrong [3] (47). The mechanism of alkaline urine

[3] Also reported by Wrong and present in our experimental data is a decrease in potassium excretion at this time, an observation of interest in view of the simultaneous formation of an alkaline urine.

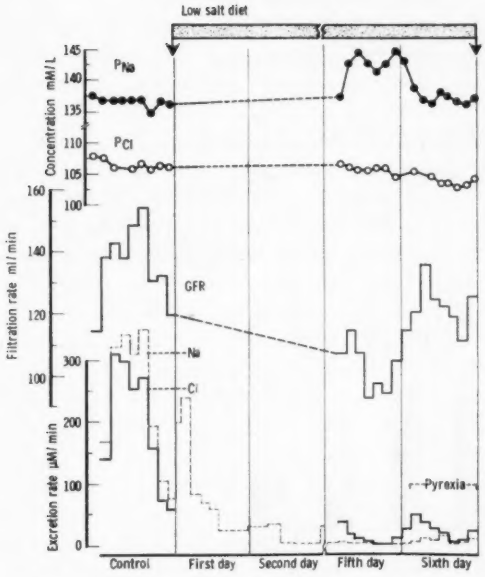

FIG. 5. DIURNAL CYCLE OF FILTRATION RATE AND SODIUM AND CHLORIDE EXCRETION BEFORE AND ON THE FIFTH AND SIXTH DAYS OF A LOW SALT DIET. Excretion of sodium, alone, was measured during the first 4 days on the low salt diet. The third and fourth days, exhibiting progressively diminishing excretion but with diurnal patterns similar to that of the second day, have been omitted as noncontributory. Subject L.W.: age 43; wt 75 kg; ht 179 cm.

formation under these circumstances is unknown. The filtration rate cycle correlates well with the sodium but not with the chloride cycle.

During the second and third experimental days, Na and Cl excretion and glomerular filtration rate (GFR) rose progressively, the latter to 138 and 167 ml per minute, compared with 102 and 117 in the control and first experimental days (Table I). The filtration rate cycle on the second day was well preserved, and its amplitude apepars increased, similar to that observed during saline infusion. Sodium and chloride excretion exceeded intake, plasma concentrations declined, weight also decreased slightly from its maximum, and frank symptoms of water intoxication ensued. Difficulty in voiding, progressing to complete retention, rendered all individual clearance periods on the third day unreliable, although the 24-hour mean value is probably accurate.

Diurnal cycle during steroidal salt retention. The diurnal cycle of NaCl excretion and glomerular filtration before and during salt retention induced by deoxycorticosterone acetate (DCA) injection is shown in Figure 3. Na and Cl excretion decreased during the first day of DCA injections but increased progressively thereafter to reach the control rate of excretion (steroid escape) on the third day. The general contour of the diurnal excretory curve appears to be little altered by DCA, as noted also by Stanbury and Thomson (12). The filtration rate cycle was not evidently affected on the first day, but both the mean values and possibly, also, the amplitude, increased progressively on the second and third days. Plasma concentrations showed little change.

Diurnal cycle during salt depletion. The diurnal cycles of NaCl excretion and glomerular filtration before and after several days on low salt diets are shown in Figure 4 and 5. Salt-free inulin was

TABLE I

Mean diurnal inulin clearance and 24-hour chloride excretion before and during alteration in body fluid balance

Protocol	Inulin clearance (ml/min)				Chloride excretion (mmoles/24 hrs)			
	Control day	Day 1	Day 2	Last day*	Control day	Day 1	Day 2	Last day*
Saline infusion (Figure 1)	128.2	131.7†	135.6	145.0	215	344	578	569
Water loading (Figure 2)	101.8†	117.3	138.5	166.6	215	85	296	420
Salt retention (Figure 3)	119.7	123.2	138.1	139.8‡	238	95	174	470‡
Low salt diet (Figure 4)	116.5			99.8	135			26
Low salt diet (Figure 5)	137.8			112.5	280			28

* Third day in experiments, Figures 1–3; fourth day in experiment, Figure 4; average of fifth and sixth days in experiment, Figure 5.

† Interpolated values are substituted for technically unsatisfactory clearance measurements in calculating a 24-hour mean value on these days.

‡ Unmeasured nighttime periods are assumed to follow the contour of the preceding cycle in calculating 24-hour mean clearance and excretion values on this day.

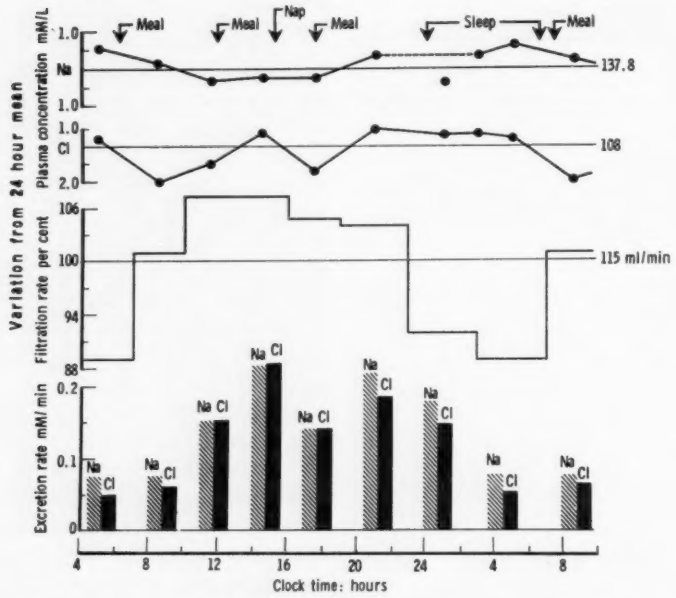

FIG. 6. AVERAGE DIURNAL CYCLE OF FILTRATION RATE, SODIUM AND CHLORIDE EXCRETION, AND PLASMA SODIUM AND CHLORIDE CONCENTRATIONS DURING CONTROL DAYS ON UNRESTRICTED DIET IN SUBJECT R.S. Filtration rate is represented as per cent variation of each period from the 24-hour mean of 115 ml per minute. Plasma sodium and chloride concentrations are represented as concentration differences of each period from the 24-hour means of 137.8 and 108 mmoles per L, respectively. Amplitudes of filtration rate and plasma sodium and chloride cycles are 21 per cent and 1 and 3 mmoles per L.

not available for one of these studies, with the result that, in Experiment 4 (Figure 4), the subject received nearly as much salt during the brief measurements of inulin clearance on the first, second, and third low salt days as he received from his diet. About 1.5 g of salt was contained in the 24-hour infusion on the fourth day. Inulin solutions employed during the second study (Figure 5) were salt-free. Although both the minimal values and the amplitude of the NaCl excretory cycles were decreased as expected, it is of additional interest that the duration of the daytime peak tended to decrease, with a corresponding widening of the nocturnal minimum.

The 24-hour mean values of glomerular filtration rate were decreased on the low salt diets from 116 to 100 and from 138 to 113 ml per minute in Subjects R.S. and L.W., respectively (Table I). The diurnal cycle of filtration rate was preserved, although the mean amplitude of both subjects (20 per cent) may have been somewhat decreased. The data are insufficient to determine whether the contour of the filtration rate cycle was altered signif antly.

DISCUSSION

A provisional description of the diurnal cycle of filtration rate in normal man can be obtained from these experiments. Filtration rate is lowest near the end of the sleeping period. It increases rapidly after awakening and, in normally hydrated individuals, remains at generally elevated levels until the beginning of the sleep period. The probable amplitude of 25 and perhaps 30 per cent of the mean value is much greater than that suggested by previous studies. Alterations in the cycle occur in normal individuals with altered body fluid balance. In subjects with expanded fluid volume as the result of saline infusion, water loading under the influence of vasopressin, or salt retention

under the influence of deoxycorticosterone, the amplitude frequently appears to be increased primarily as the result of an increase in the daytime maximum. In addition, the daytime peak tends to extend well into the sleep period. Opposite changes were observed in the subjects on a low salt diet, in whom a probable lowering of the nighttime minimum, together with a somewhat greater lowering of the daytime maximum and some decrease in amplitude, occurred. The data are insufficient to draw conclusions concerning changes in the contour of the cycle during depletion. It is of interest that a near normal cycle is maintained in the normal subject during severe salt retention, while the cycle is apparently reversed in the salt-retaining patient with heart or liver disease (25, 27).

Pertinent to interpretation of salt excretion studies is the observation that, although the 24-hour mean inulin clearance is increased during salt or water loading and decreased during salt deprivation, the diurnal cycles in all states often overlap one another. Hence, by inadvertently selecting the appropriate phase of the cycle in which to make brief measurements of filtration rate, it is possible to obtain the same value of filtration rate under widely varying conditions of fluid balance, or else varying values under constant conditions of balance. Particularly during the morning hours, random measurements of filtration rate cannot be expected to correlate satisfactorily with 24-hour measurements of sodium chloride excretion.

Correlation between filtration rate and chloride excretion in Subject R.S.,[4] during individual clearance periods in several diurnal cycles on a routine diet, is illustrated in Figure 7. The solid line is the theoretical but semiempirical relationship between filtration rate and chloride excretion as deduced from studies on the dog and transposed to man (23). The broken lines represent the uncertainty limits (2 SD, or 10 per cent) to the measurement of filtration rate. Details of the derivation of the theoretical curve are beyond the scope of this paper, but are based upon attribution to a proximal chloride transport process of a proportionality coefficient to filtration rate of 0.085 mmoles per ml, attribution to a distal chloride transport process of a maximal transport rate of

[4] Clearance data on Subjects K.B. and L.W. are insufficient for an analysis of this form.

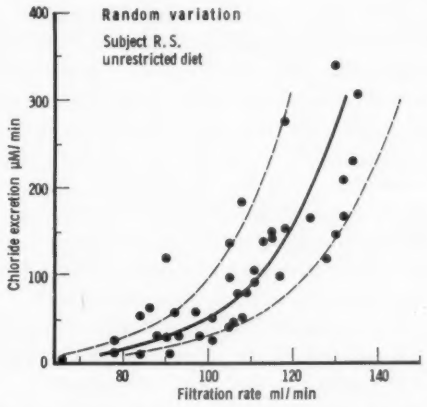

FIG. 7. CORRELATION BETWEEN FILTRATION RATE AND CHLORIDE EXCRETION DURING SEVERAL DIURNAL CYCLES IN SUBJECT R.S. ON UNRESTRICTED DIET. Each point represents an individual clearance period. The solid curve represents a hypothetical relationship between filtration rate and chloride excretion, as described in the text. The broken curves lie 2 SD (10 per cent) of estimated error of measurement of filtration from the mean value.

4.0 mmoles per minute per 1.73 m^2 and a titration splay similar to that for glucose, and existence of a filtrate chloride concentration of 107 mmoles per L. The general curve for an average man (23) is adjusted to Subject R.S. by multiplying the filtration rate scale by 0.83. All terms in the equations, including titration splay and plasma concentration, are considered significant parameters. Since the two-variable system (excretion against filtration in Figure 7) does not provide for the operation on excretion of changing plasma concentration, recourse was had to the theoretical equations. These predicted that a 1.0 mmole per L change in plasma chloride concentration should be equivalent to a 3.5 to 4.5 ml per minute change in filtration rate over the range of filtration rate values observed. Accordingly, an observed filtration rate value was "corrected" [5] if, in that clearance period, plasma chloride concentration varied from the mean concentration for all control days. The "corrected" filtration rate values are plotted in Figure 7. The correction in either direction seldom exceeded 8 or 9 ml per minute, however, and for most of the data was less than 5 ml per minute. The fit of the

[5] $GFR_{cor} = GFR_{obs} + F(P_{Cl} - \bar{P}_{Cl})$, where F is the factor converting concentration units to filtration units, averaging, in these studies, 4 ml per minute.

data to the theoretical curve is better than is warranted by the many uncertainties involved in the measurement of inulin clearance, the extension to man of constants derived under acute loading conditions on the dog, the assumption that glucose and chloride transport should have a similar titration splay, and the assumption that the relationships between plasma concentration and excretion, derived for high concentrations, obtain equally in the normal or subnormal range. It may be confidently predicted that other studies will exhibit a correlation between theory and experiment more realistically in accord with sources of error in measurements of this nature.

In view of the results of previous studies which have demonstrated a general decrease in filtration rate during dehydration or salt deprivation and an increase in filtration rate during fluid loading in normal subjects, a major objective of these experiments was determination of the time of onset and rate of change of filtration rate, and correlation of these changes with changes in NaCl excretion. In the three experiments associated with body fluid expansion, a major increase in filtration occurred on the second day of loading and, in each instance, was correlated with a marked increase in excretion above the level of the first loading day (Table I). In the two experiments on salt deprivation, filtration rate is clearly depressed below control levels by the fourth day and, as suggested by brief measurements, may well be depressed by the third or second day. It may be concluded provisionally, therefore, that changes in filtration rate closely parallel changes in salt excretion attendant upon alterations in fluid balance in healthy subjects.

Correlation between NaCl excretion and filtration rate changes do not imply that excretion normally is governed by filtration rate changes alone in an otherwise rigid filtration-reabsorption system. A contribution to excretion of filtrate concentration changes, at least within wide limits, is indicated by the effects of hypertonic saline infusions and, in the present studies, by the decrease in chloride excretion immediately after water loading (Figure 2). Susceptibility of the NaCl transport system to stimulation from various steroids is well documented (23) and is illustrated in Figure 8. Filtration rate in this subject must be roughly 25 ml per minute greater during DCA administration than in its absence, to

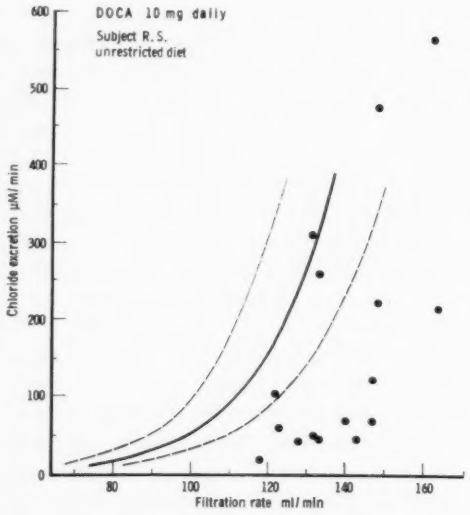

FIG. 8. CORRELATION BETWEEN FILTRATION RATE AND CHLORIDE EXCRETION DURING DEOXYCORTICOSTERONE ACETATE (DCA) ADMINISTRATION IN SUBJECT R.S. (FIGURE 3). The hypothetical curves relating excretion and filtration in the untreated state (Figure 7) are reproduced for reference. Chloride excretion rates equivalent to those observed in the untreated state are attained during DCA administration only at higher filtration rates.

maintain a constant excretion rate of 0.1 mmole per minute. The simplest description of the steroid effect, within the context of the aforementioned hypothetical transport system, is a marked decrease in the titration splay, together with a possible small increase in transport maximum, of the distal NaCl transport system. Filtration rate nevertheless would seem to account almost entirely for most short-term changes in NaCl excretion, such as are observed during the normal diurnal cycle and early after acute blood volume contraction and related stresses (23). Absence of important contributions to the NaCl excretory cycle from adrenocortical function is suggested by occurrence of near normal cycles in hypoadrenal patients (8, 20, 48, 49), together with absence of significant diurnal variations in aldosterone excretion in quiescent subjects (50–55). Rising filtration rate may well be responsible, also, for the phenomenon of steroid escape and for the delayed natriuresis which follows chronic water loading.

The mechanisms that determine the diurnal

rhythm of filtration rate and, more remotely, the changes in this rhythm under various loading conditions are unknown. That a rhythm of the magnitude observed in man is not a necessary aspect of renal function is demonstrated by the apparent absence of a significant rhythm in quiet, fasting dogs (56). The extent of pre- as compared with postglomerular vascular resistance changes that may occur during the cycle and which, therefore, constitute the immediate cause of the changes in filtration will require a more detailed analysis of simultaneous changes in renal plasma flow. Proof that the rhythm is not dependent upon the renal nerves is lacking, but no evidence of vasodilator nerves or of neurogenically controlled hemodynamic changes in unstressed animals has been discovered. By exclusion, we must postulate the existence of one or more humoral transmitters, the secretions of which are dependent, ultimately, upon a "volume receptor." No recognized hormone fulfills this function. To designate this hypothetical humoral system, which has as a major function the control of filtration rate, the term, "glomerulokinin" has been proposed elsewhere (57). In this view a humoral system controlling aldosterone secretion and a humoral system controlling glomerular filtration are two afferent arms of a reflex system controlling extracellular or vascular volumes, while the fluid volume control system is but a portion of a more general and presumably integrated system which preserves the integrity of the circulation.

SUMMARY

Filtration rate (inulin clearance) and the excretion rates and plasma concentrations of sodium and chloride were measured continuously through the diurnal cycle in three quiescent, healthy, male subjects.

The normal diurnal cycle of filtration rate under control conditions exhibits a characteristic nocturnal minimum and diurnal maximum with an amplitude of 25 and perhaps as much as 30 per cent of the mean.

Under fluid loading by saline infusion, chronic water loading supported by vasopressin injections, or salt retention induced by deoxycorticosterone acetate injections, the diurnal filtration cycle shows a tendency to a widening of the daytime maximum and a possible increase in amplitude.

Under fluid depletion attending consumption of a low salt diet, the filtration cycle shows a tendency to a decrease in amplitude and in mean level, but whether the daytime maximum is narrowed, parallel with the simultaneous narrowing of the diurnal peak in chloride excretion, cannot be determined from the available data.

In the present experiments significant changes in the 24-hour mean filtration rate level occurred relatively early, increasing on the second day of the fluid loading studies, and decreasing by the fourth day on the low salt diets.

A close quantitative correlation between filtration rate and chloride excretion is evident during the normal or control diurnal cycle of excretion, while a close qualitative correlation between excretion and filtration is evident during the excretion changes attendant upon fluid loading or depletion. A rising filtration rate is proposed as the principal cause of the delayed natriuresis occurring during sustained water loading and of the phenomenon of "steroid escape."

An unknown humoral system, provisionally termed "glomerulokinin," may determine the normal diurnal cycle of filtration rate and the changes in the cycle attendant upon alteration in body salt or fluid balance.

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DIVALENT CATION AND pH DEPENDENCE OF RAT INTRINSIC FACTOR ACTION IN EVERTED SACS AND MUCOSAL HOMOGENATES OF RAT SMALL INTESTINE *

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In 1959 it was reported in these pages (1) that hog intrinsic factor concentrate enhanced the uptake of radioactive vitamin B_{12} (Co^{60}-B_{12}) by everted sacs of rat small intestine in the cold. This enhancement was significantly reduced in the absence of calcium and was reversible to a significant degree by disodium ethylenediamine tetraacetate dihydrate (EDTA) (1), suggesting that intrinsic factor action was calcium-dependent. Disodium EDTA reduced the enhancing effect on radio-B_{12} uptake of rat intrinsic factor in loops of small intestine in a living rat; calcium EDTA was without such inhibitory effect (2). Other workers, however, threw doubt on the calcium dependence of intrinsic factor action. They reported that gastrectomized rats fed radio-B_{12} bound to rat gastric juice plus large amounts of disodium EDTA (3, 4), and normal rats fed radio-B_{12} plus disodium or tetrasodium EDTA (5), had normal radio-B_{12} absorption. These findings, taken with the fact that the *in vitro* studies did not demonstrate total loss of the effect of intrinsic factor on depletion of calcium, left the concept of the calcium dependence of intrinsic factor action in doubt.

The present report presents the results of a study aimed at resolving this uncertainty, and also at strengthening the evidence (6, 7) for the pH dependence of intrinsic factor action.

MATERIALS AND METHODS

Co^{60}-labeled vitamin B_{12} and Co^{57}-labeled vitamin B_{12}.[1] The Co^{60}-labeled vitamin B_{12} had a specific activity of approximately 1 μc per μg, and the Co^{57}-labeled vitamin B_{12} had a specific activity of approximately 5 μc per μg. The pH-dependence experiments using everted sacs were performed with Co^{57}-labeled vitamin B_{12}; all other experiments were performed with Co^{60}-labeled vitamin B_{12}.

Rat intrinsic factor concentrate (RIFC) was prepared as previously described (2).

Rat small intestine everted sac system. The homologous system (RIFC, everted sacs of rat small intestine) used by Strauss and Wilson (8–10) was employed.

Nonfasting male Sprague-Dawley rats were sacrificed. The small intestine was transected at the pylorus and 25 to 50 ml of 0.9 per cent NaCl was run through it to rinse out particulate content. The small intestine was then stripped free of its mesenteric attachment, transected at its ileal end and everted on a stainless steel rod, 112 cm long and 1.5 mm in diameter, with rounded ends. Beginning with its lower end, the rat small intestine was slipped over the rod until a silk ligature could be placed tightly over the ileal end of the intestine, and held in place by a groove in the rod 2 mm from its end. The intestine was gently and rapidly everted on itself. It was then washed by dipping repeatedly into each of two changes of 200 ml saline, and placed in a petri dish containing 0.9 per cent saline at room temperature.

Individual sacs approximately 4 cm long were prepared as described by Wilson and Wiseman (11). After being ligated at each end and slightly distended with Krebs-Henseleit bicarbonate (12) containing 200 mg per 100 ml glucose, each sac was dropped into a separate 50-ml Erlenmeyer flask containing 5 ml incubation medium plus 5,000 $\mu\mu$g of radioactive vitamin B_{12} in 0.5 ml of 0.9 per cent saline with or without added RIFC. The amount of RIFC used was prepared from 1/100 of a rat stomach, diluted with 0.9 per cent NaCl to a volume of 0.5 ml. This amount of rat stomach was chosen because it produced near maximal enhancement of vitamin B_{12} uptake in this everted sac system. Indeed, tenfold lower or higher amounts of RIFC produced markedly less enhancement, and still larger amounts inhibited radio-B_{12} uptake (data not shown).

Immediately after placing each filled everted sac in the medium, the flask was gassed for 60 seconds with 95 per cent O_2 and 5 per cent CO_2, sealed with a rubber stopper, and shaken (96 cycles per minute) at 37° C for 1 hour. At the end of this period the sac was removed and rinsed through three changes of 0.9 per cent saline. The ligated ends were then cut off, the sac cut open lengthwise and gently blotted with toilet tissue, weighed,

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[1] Kindly provided by Drs. C. Rosenblum, N. Ritter and E. Alpert of Merck Sharp & Dohme Research Laboratories, West Point, Pa.

and the quantity of its radioactivity determined in a well-type scintillation detector, for which the magnitude of the background averaged 330 counts per minute. In this detector the Co^{60}-labeled vitamin B_{12} registered approximately 1 count above background per minute per $\mu\mu$g, and the Co^{57}-labeled vitamin B_{12} registered approximately 4 counts above background per minute per $\mu\mu$g.

In all experiments with everted sacs, except those in which the site of intrinsic factor action was studied, the top and bottom 20 per cent of the rat small intestine was discarded and sacs were prepared from the remainder, beginning at its top. This was done because of the minimal-to-absent action of RIFC at top and bottom of the rat small intestine. Each experiment was repeated at least three times in order to ensure validity. Sac length was measured by placing a ruler alongside the unstretched gut, which had been allowed to retract while in 0.9 per cent NaCl at room temperature in a large petri dish.

Rat small intestine mucosal homogenate system (rat gut homogenate system). Nonfasting 200 to 300 g male Sprague-Dawley rats were sacrificed. The small intestine was transected at the pylorus, stripped free of its mesenteric attachment, and transected at its ileal end. It was then placed on a long strip of Parafilm,[2] measured, and the top and bottom 20 per cent discarded. The remaining small intestine was everted, washed free of adherent intestinal contents by dipping repeatedly into each of two changes of 0.9 per cent NaCl, and cut with scissors into roughtly 7- to 8-cm segments. Each segment was then scraped free of mucosa with two glass slides (one holding the gut in a fixed position, the other used for scraping). The mucosal scrapings from two rats were combined and placed in 25 ml of 0.9 per cent NaCl in a 50-ml beaker, shaken vigorously by hand for 10 seconds and then homogenized for 30 seconds in a Waring Blendor. Then, after being centrifuged for 10 minutes at 3,000 rpm, the liquid intermediate phase was discarded, leaving together for final suspension both the particulate material that initially floated above the liquid phase and that which sedimented. Sufficient 0.9 per cent NaCl was added to this rat small intestine homogenate so that 1 ml of the resultant suspension of homogenate could easily be pipetted into each of a series of 50-ml Erlenmeyer flasks. The homogenate prepared from one rat intestine was sufficient for 8 to 14 such flasks.

In the experiments here reported, incubation with rat gut homogenate was performed as with everted sacs, except that the gas phase was air. Freshly prepared rat gut homogenate was used, although thawed frozen homogenate is equally useful in the "assay" of RIFC (data not presented). Incubation was followed by centrifugation for 10 minutes at 3,000 rpm. The liquid phase was then removed by pipet, followed by resuspension in and centrifugation from two washes in 10 ml of 0.9 per cent NaCl containing 10 mmoles $CaCl_2$. The quantity of radioactivity retained by the sedimented rat gut homogenate was then determined in a well-type scintillation detector.

In the studies of pH dependence of intrinsic factor action, the buffer used consisted of 9 parts Krebs-Ringer solution (13), brought to appropriate pH with 1 part 0.05 M Tris-(hydroxymethyl)aminomethane-acid maleate-NaOH buffer (14). Immediately after placing each everted sac in the medium, the flasks were gassed with 100 per cent O_2 and shaken at 37° C for one-half hour. The same incubation system was used for pH-dependence

[2] Marathon Co., division of American Can Co., Menasha, Wis.

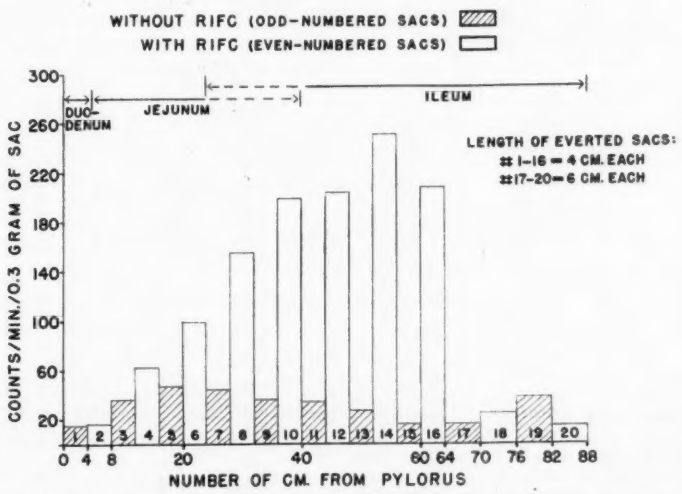

FIG. 1. ABSORPTION OF VITAMIN B_{12} AT VARIOUS LEVELS OF THE RAT SMALL INTESTINE *in vitro*, WITHOUT AND WITH ADDED RAT INTRINSIC FACTOR CONCENTRATE (RIFC).

TABLE I

*Divalent cation dependence of rat intrinsic factor concentrate (RIFC) in augmenting adsorption of Co^{60}-B_{12} by everted sacs * and mucosal homogenates of rat small intestine*

Incub. medium†	RIFC	cpm/ everted sac†	Excess counts over control sacs	cpm/ aliquot gut homogenate	Excess counts over control homogenate
Standard	0	45		264	
Standard	+	168	123	665	401
Standard − Ca‡	0	72		246	
Standard − Ca‡	+	121	49	488	242
Standard − Mg‡	0	29		231	
Standard − Mg‡	+	190	161	588	357
Standard − (Ca + Mg)‡	0	73		60	
Standard − (Ca + Mg)‡	+	92	19	53	−7
Standard	0	19			
Standard	+	307	288		

* All sacs from midportion of small intestine and about 4 cm long.
† Standard medium is Krebs-Henseleit bicarbonate, containing 200 mg % glucose and 5,000 μμg Co^{60}-B_{12}, gassed with 95% O_2-5% CO_2 (pH 7.4).
‡ Each divalent cation deleted was replaced by an equivalent millimolar concentration of sodium.

studies with rat gut homogenate, except that the gas phase was air.

RESULTS

Site of intrinsic factor action in the rat small intestine. Figure 1 demonstrates that uptake of vitamin B_{12} mediated by RIFC is greatest in everted sacs from the midportion of the rat small intestine. A relatively high Co^{60}-B_{12} uptake by the teminal ileum in the absence of RIFC was observed in three of four separate rat intestines.

Divalent cation dependence of RIFC action. Table I demonstrates that RIFC does not appreciably enhance vitamin B_{12} uptake by everted sacs of rat small intestine when both calcium and magnesium are deleted from the medium. Deletion of calcium alone appears to reduce markedly the effect of RIFC. Deletion of magnesium alone does not. The finding that deletion of calcium from the incubation medium results in higher radio-B_{12} uptake by control sacs (without RIFC) has been previously noted (1). The calcium and magnesium dependence of RIFC action is also

TABLE II

*pH dependence of rat intrinsic factor concentrate in augmenting absorption of Co^{57}-B_{12} by everted sacs * of rat small intestine*

pH of buffered medium†				
Before inc.	After inc.	RIFC	cpm/ everted sac	Excess counts over control
7.1	6.3	0	123	
7.1	6.3	+	259	136
5.7	5.7	0	102	
5.7	5.7	+	44	−58
5.7	6.8‡	0	103	
5.7	6.7‡	+	299	196
7.1	5.7§	0	86	
7.1	5.7§	+	35	−51
7.1	6.7	0	78	
7.1	6.8	+	488	410

* All sacs as in Table I; each filled with 1.1 ml buffered medium at pH 7.1.
† Buffered external medium in each flask contained 5,000 μμg Co^{57}-B_{12} with a gas phase of 100% O_2.
‡ 0.085 ml 0.2 M NaOH had been added to buffer after first 15 minutes of incubation.
§ 0.2 ml Tris acid maleate had been added to buffer after first 15 minutes of incubation.

TABLE III

pH dependence of rat intrinsic factor concentrate in augmenting absorption of Co^{60}-B_{12} by mucosal homogenate of rat small intestine

pH of buffered medium*				
Before inc.	After inc.	RIFC	cpm/ aliquot gut homogenate	Excess counts over control homogenate
4.6	4.6	0	293	
4.6	4.6	+	156	−137
5.4	5.4	0	243	
5.4	5.4	+	135	−108
5.9	5.9	0	210	
5.9	5.9	+	231	21
6.5	6.5	0	229	
6.5	6.5	+	372	143
6.9	6.9	0	236	
6.9	6.9	+	409	173
7.4	7.4	0	214	
7.4	7.4	+	406	192
7.9	7.7	0	214	
7.9	7.7	+	463	249
8.4	8.2	0	214	
8.4	8.2	+	454	240
8.9	8.6	0	221	
8.9	8.6	+	529	308

* 5,000 μμg Co^{60}-B_{12} added to each aliquot of buffered medium. Incubation performed with air as gas phase.

demonstrable in rat small intestine mucosal homogenate (Table I).

pH dependence of RIFC action. Table II shows that RIFC enhances vitamin B_{12} uptake by everted sacs of rat small intestine at pH 7.1 but not at pH 5.7. RIFC previously kept at pH 5.7 for 15 minutes at 37° C enchances vitamin B_{12} uptake by everted sacs when the pH is raised to 6.8. The enhancing effect of RIFC at pH 7.1 is reversed to one of inhibition by lowering the pH to 5.7.

Table III indicates that the enhancing effect of RIFC on vitamin B_{12} uptake by rat small intestine homogenate increases stepwise as the pH is raised stepwise from 5.9 to 8.9.

DISCUSSION

The question of relevance to physiological fact arises with all *in vitro* systems, and can only be settled in each case by demonstrating that what occurs *in vitro* also occurs in physiological circumstances *in vivo*. It has been demonstrated *in vivo* (15, 16) that the major site of absorption of vitamin B_{12} in the rat is the midportion of the small intestine. Prior studies (9, 17), confirmed here (using the entire length of rat small intestine), demonstrate that intrinsic factor-mediated vitamin B_{12} absorption in everted sacs of rat small intestine is greatest in the same portion of the small intestine. This indicates anatomical and physiological similarities of the *in vitro* everted sac system to the *in vivo* system for the absorption of vitamin B_{12} in the rat. Moreover, in quantitating the sites of vitamin B_{12} absorption, the *in vitro* system excludes *in vivo* variations in the contents of the intestinal lumen, such as distally diminished amounts of intrinsic factor and radioactive vitamin B_{12} or local differences in pH.

The finding of calcium dependence of intrinsic factor action at 37° C in everted sacs of rat small intestine and in rat small intestine homogenate using homologous intrinsic factor also has a counterpart *in vivo* (2). In man, short-term (18, 19) or long-term (19, 20) treatment with calcium-chelating agents appears to inhibit vitamin B_{12} absorption and eventually to lower serum vitamin B_{12} levels (19, 20).

Calcium appears to be essential for binding of intrinsic factor to the hypothetical receptors on the small intestinal mucosa (1, 2). It is interesting that in Hydra, which requires bound calcium for the action of surface receptors, magnesium antagonizes rather than partially substitutes for the action of calcium (21). The finding that magnesium may partially substitute for calcium was foreshadowed by the evidence that magnesium EDTA was a less potent blocker of RIFC action than was disodium EDTA (2).

The studies of pH dependence here reported reinforce previous clinical (6) and experimental (7) findings *in vivo* and *in vitro*. They demonstrate that RIFC is unable to enhance vitamin B_{12} absorption at pH 5.7, but that a slight rise in pH to 5.9 or above allows such enhancement. The suppression of ionization of carboxyl groups on mucosal cell surfaces (22) or on mucoproteins (23) by low pH may be related to the dependence of RIFC adsorption on divalent cations such as Ca^{++} and Mg^{++}. At any rate, it appears that the ineffectiveness of RIFC at pH 5.7 is *not* due to damage to the molecule, since its enhancement ability is restored on again raising the pH. These findings suggest that a pH above 5.7 is required for intrinsic factor to attach to receptors on the intestinal mucosa, especially since the enhancing effect of RIFC on vitamin B_{12} uptake by everted sacs of rat small intestine appears to become inhibitory when the pH of the system is reduced to 5.7. This may be due to a combined effect: the low pH obliterates the RIFC enhancement and RIFC binds most of the vitamin B_{12} (2), leaving very little free in solution to diffuse into the gut mucosa.

Preliminary experiments (not shown) demonstrated that comparable enhancing effects with RIFC upon Co^{60}-B_{12} absorption could be demonstrated in either everted sacs or suspensions or homogenates of mucosal scrapings from the rat small intestine. The homogenate system possesses the advantage that it eliminates variations in the measurement or in the inherent activity of the mucosa of different everted mid-intestinal segments (Figure 1). Consequently, a single aliquot of the mucosal homogenate can serve as a control for a number of experimental flasks. In this respect the intestinal homogenate system has the advantages for RIFC studies that the liver homogenate system (24) possesses for studies (24, 25) of hog intrinsic factor concentrate activity. In the intestinal homogenate system, as in

the liver homogenate system, the intrinsic factor effect is observable at room temperature and at 3° C, as well as at 37° C (data not presented). Since each aliquot of intestinal homogenate is identical with every other aliquot, this system appears to provide a fairly quantitative assay for intrinsic factor activity. Unpublished studies (26) indicate that homogenate prepared from the bottom half of guinea pig small intestine may prove more useful for "assay" of human intrinsic factor activity than the everted sacs of guinea pig small intestine previously (10) suggested.

SUMMARY

1. Prior *in vivo* and *in vitro* findings that the midportion of the small intestine of the rat is the site of vitamin B_{12} absorption were confirmed *in vitro*.

2. Calcium is required for rat intrinsic factor concentrate (RIFC) adsorption to everted sacs of rat small intestine or to rat small intestine mucosal homogenate *in vitro*. Magnesium can partially substitute for calcium.

3. The enhancing effect of RIFC on vitamin B_{12} absorption occurs at pH 5.9 or higher. It is sharply pH dependent and at pH 5.7 is reversed to an inhibitory effect.

4. Small intestine mucosal homogenate is a useful tool for studies of intrinsic factor activity and the factors affecting it.

ADDENDUM

At the Second European Symposium on Vitamin B_{12} and Intrinsic Factor (Hamburg, Germany, August 2–5, 1961) (Proceedings to be published by Ferdinand Enke Verlag, Stuttgart) it was reported by Niewig, Abels, Vegter and Hellemans that sodium bicarbonate enhanced the decreased vitamin B_{12} absorption of two patients with pancreatic insufficiency, presumably by raising the low pH of the duodenal contents. Their results suggest that our findings in rats *in vitro* may be applicable to man *in vivo*.

ACKNOWLEDGMENT

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USE OF NEUTRON ACTIVATION ANALYSIS FOR STUDYING STABLE IODIDE UPTAKE BY THE THYROID *

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Measurement of the rate of accumulation of radioiodine by the thyroid gland is of considerable clinical value, since in most instances an avid uptake of radioiodine may be correctly assumed to be due to a high rate of uptake of stable iodide by the thyroid. This measurement, expressed as a percentage of the administered radioiodine dose accumulated per unit time by the thyroid, does not give a quantitative measurement of the stable inorganic iodide being accumulated by the gland. Occasionally an avidity for radioiodine may be attributable to an abnormally small extrathyroidal iodide pool, as in the case of the patients with endemic goiter described in the classic report of Stanbury and his associates (1).

In 1949 Stanley described a method for the measurement of stable iodide accumulation by the thyroid gland using the radioiodine uptake of the thyroid and the specific activity of simultaneously excreted urine; i.e., the ratio of radioactive to stable iodide in the urine (2). The technique extended the information obtainable by the study of radioiodine accumulation alone. Technical difficulties associated with the chemical measurement of trace quantities of stable inorganic iodide have prevented its widespread application to studies of thyroid disease. The purpose of this paper is *1*) to describe a new technique for the accurate and sensitive measurement of iodide in biological fluids, and *2*) to present the results of its use in studies of the relationship between plasma inorganic iodide concentration and iodide accumulation by the human thyroid gland.

METHODS

Principles of activation analysis. Since its introduction by Hevesy in 1935, neutron activation analysis has been developed into one of the most sensitive and accurate methods for the determination of trace elements. The element to be determined is made radioactive by neutron irradiation, usually carried out in a nuclear reactor, and is identified by the characteristic gamma-ray emission and rate of decay of the resulting radionuclide. Quantitative measurements are possible, since the amount of radioactivity induced is proportional to the amount of the element present.

In some tissues trace elements are sufficiently concentrated to be measured without chemical separation prior to activation. For example, gamma-ray spectra of the radioactivity induced in human tooth, thyroid, and bone are shown in Figure 1. The gamma spectrum of tooth after 1 minute's activation was very similar to the spectrum obtained from activation of ammonium fluoride. The photopeak energy (Figure 1A) and the half-life were characteristic of fluorine[20]. The gamma spectrum of human thyroid tissue included the characteristic photopeak of iodine[128] and had an identical half-life (Figure 1B). Figure 1C shows the result of 10-minute activation of normal human bone. The gamma spectrum had the photopeaks of calcium[49], fluorine[20], sodium[24], and magnesium[27].

In most biological materials it was not possible to detect trace elements by neutron activation without removing the large quantities of sodium and chlorine that dominated the gamma-ray spectrum. Spencer, Mitchell and King (3) presented the results of neutron irradiation of serum. Gamma emissions from Na^{24} and Cl^{38} prevented application of the technique to studies of trace elements in serum. Similarly, activation of normal urine for 1 hour produced a gamma-ray spectrum identical with that of sodium chloride (Figure 2). Utilization of differential activation times or differential decay times to measure iodide in the presence of large amounts of chloride was impossible because of the similar half-lives of chlorine[38] (37 minutes) and iodine[128] (25 minutes); to measure iodide (or bromide) in urine, it was necessary to separate these halogens from chloride and sodium prior to activation.

Analytical procedure for measurement of urinary iodide. Urine samples that had been collected in iodine-free containers were passed at a flow rate of approximately 100 ml per hour through polyethylene columns (5 cm long, 0.6 cm diameter) containing 0.8 ml of Amberlite IRA-400 anionic exchange resin (Cl^- form). Urinary iodide was adsorbed by the column, while sodium and other cat-

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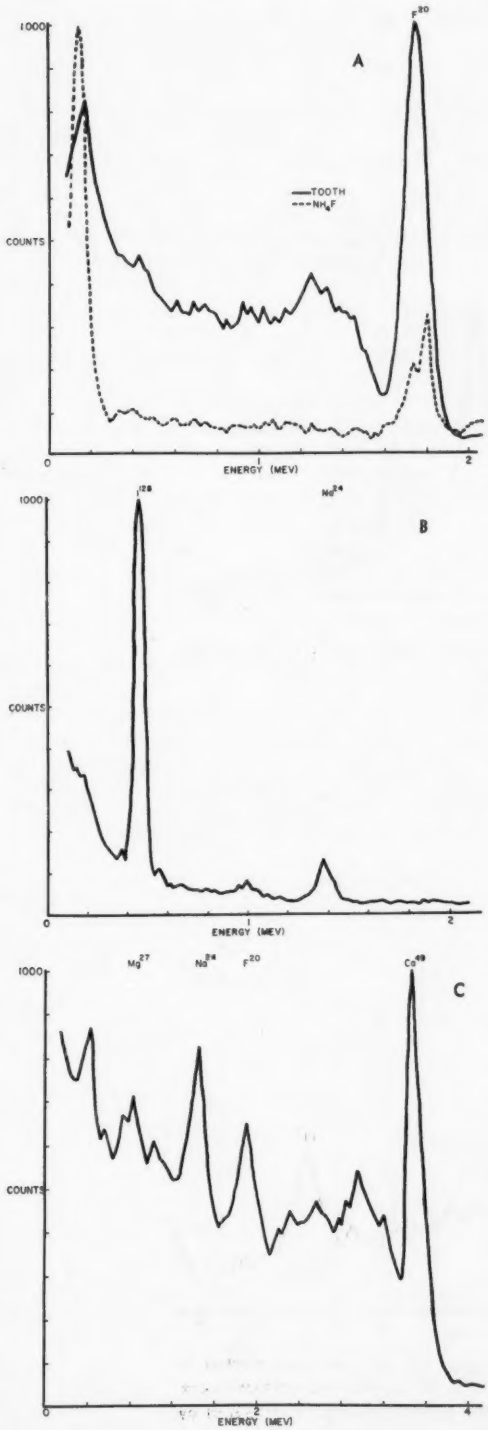

ions passed through. The chloride on the resin column was then preferentially eluted with 60 ml of 0.013 M ammonium thiocyanate solution. At this volume and concentration, thiocyanate was able to displace chloride without displacing iodide from the resin. With iodine^{131} or iodine^{132} as tracer, the recovery of iodide from this procedure was determined for each sample studied. The resin columns were then wrapped in plastic film to prevent contamination. After the radioiodine had decayed sufficiently,[1] the resin columns were activated for 1 hour in the National Naval Medical Center reactor at a thermal neutron flux of 2.25×10^{13} neutrons per cm^2 per second.

After activation the samples were placed in a scintillation detector, a 2.75-inch diameter well-shaped sodium iodide crystal. Gamma-ray spectra were determined on a 100-channel pulse height analyzer. A typical spectrum of a urine sample after activation is shown in Figure 3. The photopeaks are those of iodine^{128}, bromine^{80} and residual chlorine^{38}. The resin blank and the polyethylene container showed negligible induced radioactivity at this neutron flux. It can be seen (Figure 3) that the Compton radiation from the residual chlorine^{38} and bromine^{80} contributed to the radioactivity measured at the iodine^{128} photopeak. Therefore it was necessary to subtract this activity to obtain the true count rate of iodine^{128}. With each unknown sample, the amount of iodide on the resin column was calculated by comparing its induced activity with the activity induced in iodide standards of known weight activated on identical resin columns under identical conditions. The relationship between induced iodine^{128} radioactivity and the iodide content of standard solutions of ammonium iodide in a typical experiment is shown in Figure 4. There was a linear response of 37 cpm per μg iodide. This induced radioactivity was very reproducible ($\pm$ 5 per cent) and agreed with the radioactivity predictable for the neutron flux and the detection system employed.

Experimental procedure used to measure iodide uptake by the thyroid. The procedure used in patients to measure the urinary excretion and thyroid uptake of stable iodide was as follows. The studies were begun at 9 a.m. with the patients in the fasting state. After the patient had emptied his bladder, a tracer dose of 4 μc of carrier-free sodium iodide^{131} or 10 μc of carrier-free sodium iodide^{132} was given. Both the oral and intravenous routes of administration were studied. At the end of 2 hours thyroid uptake was measured with a flat-field collimated 2.75-inch sodium iodide scintillation detector, gamma-ray

[1] I^{132} was more suitable than I^{131}, since the shorter half-life of 2.3 hours permitted activation 24 hours after collection of the urine.

FIG. 1. **A.** GAMMA-RAY SPECTRUM OF HUMAN TOOTH COMPARED WITH THAT OF AMMONIUM FLUORIDE. **B.** GAMMA-RAY SPECTRUM OF HUMAN THYROID; I^{128} IS THE PRINCIPAL PHOTOPEAK OF THE SPECTRUM. **C.** GAMMA-RAY SPECTRUM OF HUMAN BONE; PHOTOPEAKS ARE Ca^{49}, F^{20}, Na^{24}, AND Mg^{27}.

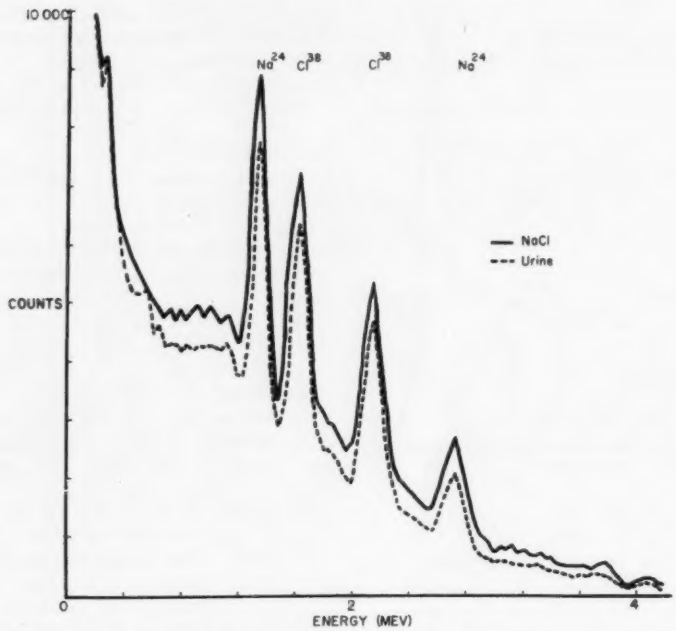

FIG. 2. GAMMA-RAY SPECTRUM OF URINE IS SIMILAR TO THAT OF SODIUM CHLORIDE.

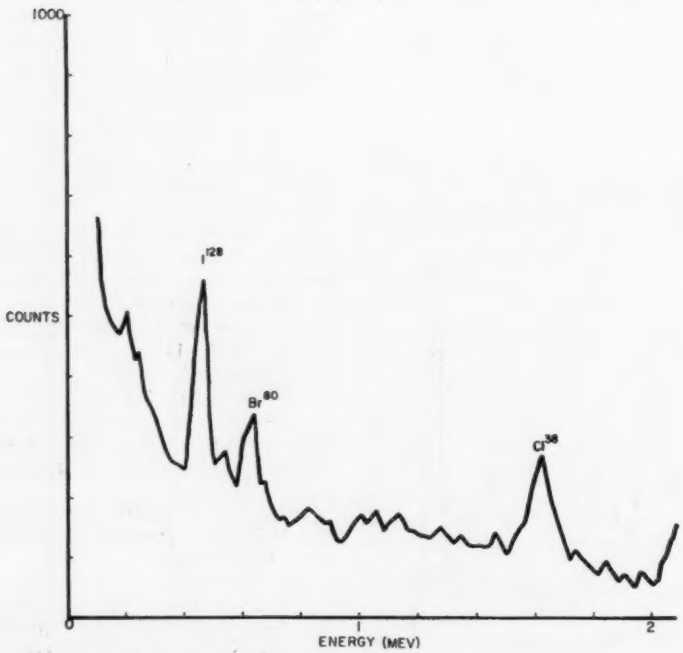

FIG. 3. GAMMA-RAY SPECTRUM OF A RESIN COLUMN AFTER ADSORPTION OF URINE AND ELUTION WITH 60 ML OF 0.013 M AMMONIUM THIOCYANATE. The photopeaks are iodine[128], bromine[80], the residual chlorine[38] not eluted by the thiocyanate.

spectrometer, and ORINS standard thyroid uptake phantom. The thyroid uptake was expressed as

$$\frac{\text{neck radioactivity} - \text{radioactivity with thyroid shielded}}{\text{dose radioactivity in standard phantom} - \text{radioactivity with dose in phantom shielded}} \times 100$$

In I^{131} studies, a 1.5 cm-thick lead shield was used to eliminate the thyroidal radioactivity; a 5 cm-thick shield was used in I^{132} studies because of the increased gamma energy from the latter nuclide.

A further correction for extrathyroidal radioactivity was determined by studies in which 10 euthyroid subjects were given large doses of Lugol's solution to completely block thyroid uptake. Neck measurements were made 2 hours after the intravenous injection of I^{131} and I^{132} (five subjects for each isotope). The radioactivity counted beneath the lead shield was 2.6 ± 0.4 per cent dose (mean $\pm$ 1 SD) for I^{132} and 2.5 ± 0.8 per cent dose for I^{131}. These values were subtracted from the thyroid uptake measurements in calculation of the uptake rates of stable iodide.

Immediately after the thyroid measurement the patient urinated into a disposable iodine-free container. The collected urine was then passed through one of the previously described resin columns. Recovery of iodide by the resin column was measured, with the I^{131} or I^{132} as a tracer. The percentage of the administered dose of radioiodine on the resin column was measured by comparing it with a standard containing one-tenth of the dose adsorbed by a blank resin column and counted under identical geometric conditions.

After the administered radioiodine had decayed to negligible levels, the stable iodide content of each resin column was measured as described previously. The thyroid uptake of stable iodide was calculated according to the following formula:

$$\text{thyroid uptake (TU) } (\mu g/2 \text{ hrs}) = \frac{\text{TU (\% dose } I^{131}/2 \text{ hrs)} \times \text{urine iodide excretion } (\mu g/2 \text{ hrs})}{\text{urine excr.}_{\blacktriangle} (\%_{\blacktriangle} \text{dose } I^{131}/2 \text{ hrs})}$$

In five subjects selected as euthyroid, the relationship between plasma inorganic iodide concentration and thyroid iodide accumulation was measured at various plasma inorganic iodide levels produced by exogenous iodide administration. These subjects were hospitalized on the research ward of the Johns Hopkins Hospital, and received a usual hospital diet. After control studies iodine was given orally every 4 hours as potassium iodide. The daily dose was increased progressively over a 2-week period from 1,200 to 7,200 μg per day. In three subjects iodide administration at a level of 10,000 μg per day was continued for an average of 37 days. Radioiodine accumulation was measured daily for 12 days; thereafter it was measured weekly. Radioiodine (I^{132}) was given intravenously 2 hours after the 8 a.m. oral iodide dose. Measurement of stable and radioactive urinary iodide was performed as previously described.

Plasma iodide concentration 1 hour after administration of the dose was measured by comparing the radioactivity of the plasma with a standard containing 1/25 of the dose. The sample was obtained at the midpoint of the urine collection period. The plasma iodide concentration was calculated, using the urinary specific activity as follows:

$$\frac{\%\text{ dose } I^{132}/5 \text{ ml plasma}}{\mu g\ I^{127}/5 \text{ ml plasma}} = \frac{\%\text{ dose } I^{132} \text{ urine}}{\mu g\ I^{127} \text{ urine}}$$

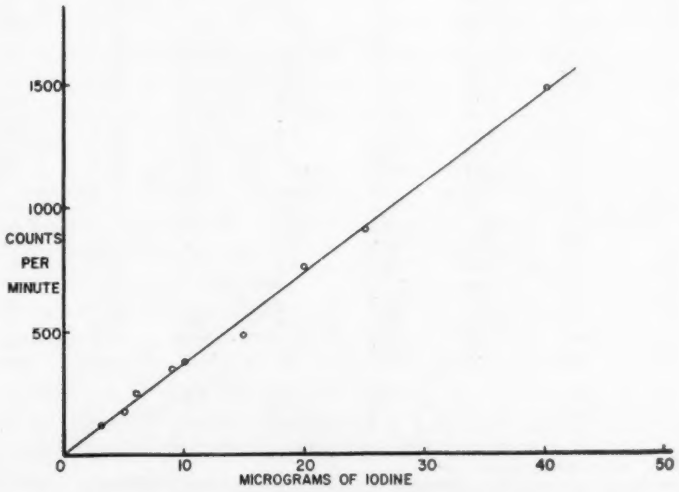

FIG. 4. RELATIONSHIP BETWEEN IODIDE CONTENT (NH_4I STANDARDS) AND INDUCED RADIOACTIVITY AS MEASURED IN THE PRESENT SYSTEM.

or

$$\frac{\mu g\ I^{127}}{5\ ml\ plasma} = \frac{\mu g\ I^{127}\ urine \times \%\ dose\ I^{128}/5\ ml\ plasma}{\%\ dose\ I^{128}\ urine}$$

RESULTS

I. Study of euthyroid subjects. Thirty-four euthyroid subjects were studied (Table I). The mean uptake of stable iodide corrected for total neck extrathyroidal radioactivity was 5.9 ± 4.4 (mean $\pm$ SD) μg per 2 hours (range, 1 to 20 μg per 2 hrs). The mean excretion of stable iodide was 16.9 ± 9.4 μg per 2 hours (mean $\pm$ SD). The radioiodine uptake ranged from 4 to 13 per cent per 2 hours. In 15 euthyroid subjects the plasma concentration of stable iodide was determined 1 hour after administration of the tracer dose. The mean value was 5 μg per L (range, 1 to 10 μg per L).

Of particular interest was the finding that the subjects with the highest excretion of stable iodide did *not* have a radioiodine uptake significantly different from those with lower urinary iodide excretion rates. In addition, over the range of plasma iodide concentration observed in these

TABLE I

Stable and radioiodine measurements in euthyroid subjects

Subj.	Urine excret.	Urine excret.	Urine spec. activ.	Thyroid uptake*	Thyroid uptake†	Thyroid uptake	Extrathy. iodide pool‡	Extrathy. iodide pool	Plasma inorg. iodide
no.	*% dose/ 2 hrs*	*μg I^{127}/ 2 hrs*	*μg I^{127}/ 1% dose radio-iodine*	*% dose/ 2 hrs*	*% dose/ 2 hrs*	*μg I^{127}/ 2 hrs*	*% dose*	*μg I^{127}*	*μg I^{127}/L*
1	28.4	22.3	0.78	6.0	3.5	2.4	83.0	64.7	
2	14.2	8.0	0.56	8.0	5.5	4.5	92.0	51.5	
3	13.2	8.8	0.67	4.3	1.8	3.7	91.5	61.3	
4	15.4	10.8	0.70	6.0	3.5	2.4	89.5	62.6	
5	20.8	9.2	0.44	5.7	3.2	1.4	88.0	38.8	
6	20.2	16.6	0.81	5.2	2.7	2.2	88.5	71.6	
7	9.6	18.7	1.95	9.0	6.5	12.7	92.0	179.2	
8	8.4	16.3	1.94	4.3	1.8	3.5	94.9	184.0	
9	13.0	35.4	2.71	9.7	7.2	19.5	88.9	241.9	
10	17.1	30.0	1.75	6.5	4.0	7.0	89.4	156.5	
11	24.3	17.3	0.72	5.2	2.7	1.9	86.5	62.3	
12	22.8	20.6	0.91	9.2	6.7	6.0	85.2	76.6	
13	13.3	26.8	2.02	6.8	4.3	8.7	91.2	184.0	
14	29.8	31.9	1.07	4.1	1.6	1.7	84.2	90.0	
15	23.1	8.2	0.35	8.6	6.1	2.1	85.4	29.8	
16	18.0	28.2	1.57	6.6	4.1	6.4	89.0	139.5	
17	12.2	29.2	2.40	5.9	3.4	8.2	91.2	218.2	
18	6.8	2.0	0.29	7.9	5.4	1.6	93.9	27.2	2.0
19	22.9	16.8	0.73	9.6	7.1	5.2	85.0	62.3	
20	9.4	10.3	1.10	7.5	5.0	5.5	92.8	102.0	
21			1.86	10.7	8.2	15.2			10.0
22	14.2	14.2	1.00	8.1	5.6	5.6	90.1	90.1	5.0
23	22.6	6.0	0.26	7.8	5.3	1.5	86.0	22.7	1.0
24	7.8	4.5	0.57	13.0	10.5	6.0	91.8	52.2	6.0
25	5.0	7.1	1.42	5.9	3.4	4.7	95.8	137.5	7.0
26	10.9	20.7	1.90	9.6	7.1	7.3	91.0	93.9	9.0
27	13.8	9.8	0.71	9.0	6.5	4.6	88.8	63.0	4.0
28	12.3	12.7	1.03	6.0	3.5	3.6	92.1	95.0	8.0
29	21.0	23.3	1.11	6.3	3.8	4.2	87.6	97.1	8.0
30	21.7	23.0	1.06	11.5	9.0	9.4	84.6	89.7	7.0
31	16.0	6.5	0.41	11.0	8.5	3.5	87.7	35.8	2.0
32	17.3	9.9	0.57	8.9	6.4	3.6	88.1	50.3	4.0
33	16.0	16.9	1.02	10.4	7.9	8.1	88.1	90.0	4.0
34	22.2	36.2	1.63	12.4	9.9	16.2	84.0	136.5	5.0
Mean	16.5	16.9		7.8	5.4	5.9		95.7	5.0
SD	6.3	9.4		2.4	2.3	4.4		56.8	3.0

* Corrected by neck minus shielded-neck method.
† Additional correction by thyroid block for total neck extrathyroidal radioactivity.
‡ Calculated as follows:
100% dose radioiodine $-$ [$\frac{1}{2}$ thyroid uptake (% dose/2 hrs) $+$ $\frac{1}{2}$ urine excretion (% dose/2 hrs)].

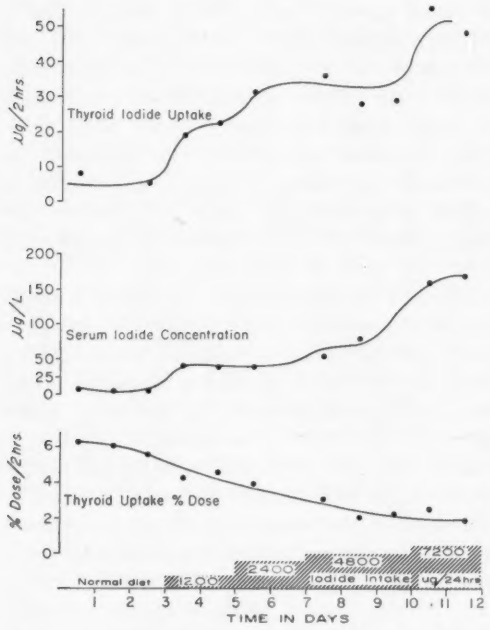

FIG. 5. MEASUREMENTS OF ACCUMULATION OF I^{131} AND I^{132} BY THE THYROID DURING THE FIRST 10 DAYS OF INCREASED IODIDE INTAKE IN A EUTHYROID SUBJECT. Plasma inorganic iodide concentration is shown.

subjects, stable iodide uptake by the thyroid was highest in those subjects with the highest concentration of inorganic iodide in their plasma. As a result of these findings, the study was extended as described in the following section.

II. Relationship between serum iodide level and the accumulation of iodide by euthyroid subjects. Selected euthyroid subjects were hospitalized and received a usual hospital diet. After control studies iodine was given orally every 4 hours as potassium iodide. The daily dose was increased progressively over a 2-week period from 1,200 to 7,200 μg per day. In three subjects iodide administration at a level of 10,000 μg per day was continued for an average of 37 days. Stable and radioiodine accumulation was measured daily for 12 days; thereafter it was measured weekly.

An example of the first 12 days of a typical study is presented in Figure 5. During the control period, when no exogenous iodide was added to the diet, the patient's 2-hour thyroid uptake (per cent dose) was 6 per cent on successive days. The patient excreted 15 and 14 μg of inorganic iodide per 2 hours. The thyroid uptake of stable iodide was 8 and 5.5 μg per 2 hours. Corresponding plasma concentrations were 7 and 5 μg per L. During administration of potassium iodide, begun on the fourth day, the uptake of radioiodine (I^{132}) by the thyroid gradually decreased. By Day 12 it had decreased to 1.9 per cent dose per 2 hours. Concurrently, the urinary excretion of I^{127} increased progressively to 418 μg per 2 hours. In contrast to the decreasing radioiodine uptake, the uptake of stable iodide increased from 7 μg per 2 hours during the control period to 42 μg per 2 hours on Day 12. The plasma inorganic iodide concentration increased to 169 μg per L.

Thus it was found that, as the iodide intake increased from 1,200 to 7,200 μg per day, the thyroid uptake of radioiodine decreased. However, the concomitant increase in plasma iodide concentration resulted in a progressive accumulation of stable iodide despite the decreased radioiodine accumulation.

Each of the five euthyroid subjects studied during iodide administration showed a similar pattern during the first 2 weeks. A summary of the results in the five patients is presented in Figure 6. At each level of iodide intake, extending over the entire period of 37 days, the thyroid uptake is shown both as a percentage of the radioiodine dose and as uptake of stable iodide. Despite the fall in radioiodine accumulation as the iodide intake increased, iodide accumulation increased rapidly until the intake reached 5,500 μg per day. Thereafter it fell steadily, but even after more than a month of high iodide intake, stable iodide accumulation exceeded control levels. In one subject the uptake of stable iodide was 75 μg per 2 hours while receiving 7,200 μg iodide per day.

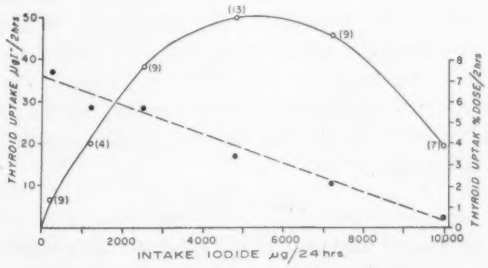

FIG. 6. RELATIONSHIP BETWEEN IODIDE ADMINISTRATION AND THYROID UPTAKE OF STABLE IODIDE DURING THE 37-DAY PERIOD OF IODIDE ADMINISTRATION.

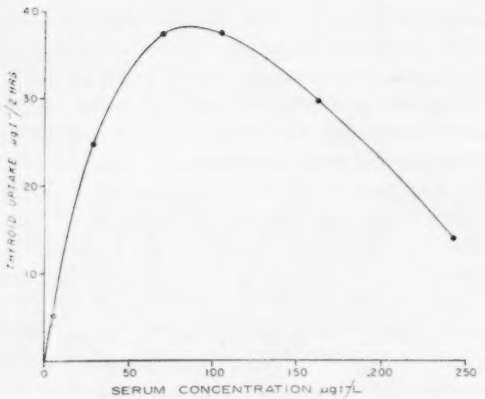

FIG. 7. RELATIONSHIP BETWEEN PLASMA INORGANIC IODIDE CONCENTRATION AND UPTAKE OF STABLE IODIDE BY THE THYROID. The study covered a period of 37 days.

On Day 16 of the study, while on this dose of iodide, the subject was given 20 μc of I^{131}. The 2-hour radioiodine uptake (corrected for neck extrathyroidal radioactivity) was 3 per cent; 48 hours later 10 per cent of the administered dose remained in the gland. This indicates that the inorganic iodide continued to accumulate during the 2-day period and that iodide initially trapped was not being readily discharged from the gland. This does not imply that synthesis of the trapped iodide was progressing to thyroid hormone, but that the trapped iodide was not merely entering the thyroid and shortly thereafter re-entering the circulation.

The plasma concentration of stable iodide was determined at each level of iodide administration in all five subjects. A total of 51 determinations was made. Figure 7 illustrates that the initial increase in concentration of plasma iodide produced a corresponding increase in the rate of uptake of stable iodide by the thyroid. After the plasma iodide concentration reached 100 μg per L, iodide accumulation began to fall. However, even at the end of the 37-day period of the study, uptake of stable iodide by the thyroid still greatly exceeded control values.

DISCUSSION

The present studies were designed to enable measurement of the rate of uptake of stable iodide by the thyroid in micrograms per unit time. A technique of neutron activation analysis was developed to enable accurate and sensitive measurement of the trace element, iodide, in biological fluids. In the past, application of neutron irradiation techniques to the analysis of trace elements in biological material has been limited by the large quantities of sodium and chloride that dominate the gamma-ray spectrum. The present technique utilized anion exchange resins and selective elution to remove these substances prior to activation.

At the neutron flux employed (2.25×10^8 n/cm^2/sec) although it was not possible to measure the exceedingly small amounts of inorganic iodide normally present in plasma, urinary iodide concentration could be readily measured. Our basic assumption in measuring the stable iodide uptake by the thyroid was identical with that of Stanley (2), who measured both the radioiodine uptake of the thyroid gland and stable iodide concentration of the simultaneously excreted urine. At any moment after the administration of a tracer dose of radioiodine, the specific activity (I^{131}/I^{127}) of urinary iodide is the same as that of plasma. This assumption appears justified, since there is no evidence of a renal iodide pool (4). Furthermore, during an experiment in which exogenous iodide was given and plasma iodide was raised to measurable levels, the simultaneous specific activities of serum and urine were nearly identical.

In 34 fasting euthyroid subjects, the mean iodide uptake was 6 ± 4 μg per 2 hours (mean $\pm$ SD). These values were lower than those of Stanley (2) who found that mean thyroid uptake of iodide in 14 subjects was 10 μg per hour (range, 3 to 19), and of Fitting (5) who found that mean uptake of eight fasting euthyroid subjects was 4.5 μg per hour (range, 1 to 17). More recently, Koutras and co-workers (6) reported the mean iodide uptake of eight euthyroid patients to be 2.9 ± 1.3 μg per hour (mean $\pm$ 1 SD). In the euthyroid subjects of the present study the radioiodine uptake did not correlate with urinary iodide excretion or the plasma concentration of inorganic iodide. Thus, in these subjects, living in an area where there was no iodine deficiency, the *radioiodine* accumulation by the thyroid did not appear measurably influenced by the day-to-day fluctuations in iodide pool size. Similar results were reported in the normal subjects without evidence of iodide deficiency studied by Koutras and

associates. The correlation between radioiodine accumulation and stable iodide concentration in patients with nontoxic goiter was observed only when the serum iodide concentration was below 1 to 2 μg per L.

Childs and collaborators reported in 1950 that in hyperthyroid subjects, the iodide-trapping function as measured by radioiodine accumulation was not altered with carrier iodide doses in the range 0.1 to 100 mg, but was suppressed by doses of 500 mg or more (7). In contrast, the synthesis of thyroid hormone by the gland was suppressed at lower concentrations (5 μg of inorganic iodide per 100 ml plasma) than those required to suppress the iodide-trapping function. This work extended to hyperthyroid patients the studies of Wolff and Chaikoff who had found that organic binding of iodide by the rat thyroid was blocked so long as plasma iodine remained above 20 to 35 μg per 100 ml (8). However, despite the block in organic binding of iodide, the rat thyroid continued to concentrate iodide.

To study experimentally the effect of large increases in iodide pool size on both radioiodine uptake and the accumulation of stable iodide by the thyroid, five euthyroid subjects were given progressively increasing amounts of oral iodide. As shown in Figure 7, radioiodine uptake decreased as iodide intake increased, but the rate of decrease of radioiodine uptake was very gradual. Consequently, the thyroid accumulated increasing quantities of stable iodide. When iodide intake exceeded 6,000 μg per day, the radioiodine accumulation had decreased to the point that the amount of *stable* iodide accumulated began to decrease despite increasing iodide intake. At the end of 37 days, however, *stable* iodide accumulation by the thyroid still exceeded control levels.

To explain our observations, we suggest the following hypothesis. The thyroid gland accumulates a constant fraction of the extrathyroidal iodide pool; i.e., iodide accumulation approximates a first-order reaction. Thus, day-to-day variations in iodide intake result in corresponding variations in the *amount* of stable iodide accumulated by the thyroid. If we postulate that the iodide concentration gradient in the thyroid gland is maintained by the presence of a carrier with which iodide reacts, how can the first-order dynamics be explained? We must postulate that the concentration of the carrier is high relative to the iodide concentration in plasma. It is not significantly reduced by its reaction with plasma iodide; consequently, it can be treated as a constant.

Variation in the rate of the first-order reaction —i.e., in the rate of accumulation of radioiodine— is produced by physiological increases or decreases in the concentration of the carrier. For example, prolonged administration of iodide results in a gradual decrease in radioiodine accumulation, presumably until stable iodide accumulation is reduced to normal amounts. This can be seen in the present experiments and is consistent with the clinical observation that prolonged iodide administration does not result in hyperthyroidism. On the other hand, the classic studies of Stanbury and, more recently, of Koutras and co-workers, are consistent with the hypothesis that prolonged iodide deficiency eventually results in an increased amount of carrier substance. This results in an increased rate of the first-order reaction describing iodide accumulation. Consequently, many iodide-deficient patients accumulate normal amounts of iodide and remain euthyroid. That these patients also accumulate iodide by a reaction with first-order dynamics is suggested by the observation of Stanbury that the administration of 1,500 μg of stable iodide daily for as long as a week failed to change the radioiodine uptake.

SUMMARY AND CONCLUSIONS

1. In the present study a technique of neutron activation analysis was used to measure trace quantities of inorganic iodide in urine. Anion exchange resins and selective elution permitted removal of interfering sodium and chloride from urine prior to the activation procedure.

2. The uptake of stable iodide by the thyroid was determined by measuring the radioiodine uptake and specific activity (I^{131}/I^{127}) of simultaneously excreted urine.

3. In 34 fasting euthyroid subjects the mean iodide uptake by the thyroid was 3 ± 2 μg per hour. Plasma concentration of inorganic iodide averaged 0.005 μg per ml.

4. Day-to-day variations in iodide intake resulted in corresponding variations in the *amount* of stable iodide accumulated by the thyroid, since iodide accumulation approximated a first-order reaction.

5. Prolonged administration of exogenous iodide resulted in a gradual decrease in radioiodine accumulation. This resulted in a decreased accumulation of *stable* iodide only after several weeks of greatly increased iodide uptake by the thyroid.

6. A mechanism postulated to explain the dynamics of iodide accumulation by the thyroid was presented.

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THE SPECIFIC STRUCTURE OF COMPOUNDS EFFECTING FAT MOBILIZATION IN MAN *

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Both epinephrine and norepinephrine enhance the process of fat mobilization. This property of promoting lipolysis has been observed both *in vitro* by demonstrating the release of free fatty acids (FFA) and glycerol from adipose tissue within the medium, and *in vivo* by the increase in the levels of plasma FFA in the fasted, resting individual (1–3). It was the intent of this study to determine whether the property of lipolysis was a singular characteristic of epinephrine and norepinephrine or whether other catechols and other vasoactive substances also could promote fat mobilization. It is known that a certain structure-activity relationship exists for the various sympathomimetic compounds as well as for other classes of drugs. It was of interest to determine whether lipolysis was also dependent upon a specific chemical structure.

METHODS

Volunteer male subjects or patients without known metabolic disorders were studied after an overnight fast. After being brought to the laboratory the individual was placed in the supine position. Cournand needles were inserted into antecubital veins of both arms, and all effort was made to reduce conversation or external stimuli. A series of two to three preinfusion ("control") samples was then drawn 10 to 15 minutes apart. A 15-minute period of infusion of the test substance was then begun, with a Bowman constant infusion pump. After the infusion, samples were drawn at 0, 10, 20, 30, 45, and 60 minutes. Blood pressure was measured by the cuff technique. The total period of observation did not exceed 120 minutes from the time the subject entered the laboratory until the last samples was drawn. All blood samples were drawn into heparinized syringes and immediately chilled. Plasma FFA was measured by a modification of the Dole technique (4).

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RESULTS

The initial studies were directed at developing a "standardized" or "control" method of assay of compound activity. The first set of observations was made on 20 fasting subjects who were kept in the supine, resting position for the entire study, receiving an intravenous infusion of 0.9 per cent saline. The mean ($\pm$ 1 SE) peak rise in plasma FFA (when compared with the immediate preinfusion value) for this group was 150 ± 42 μEq per L. This method of assessing the change in plasma FFA level was used for all studies. The second set of observations was designed to determine the response to both norepinephrine and epinephrine when administered in amounts that do not produce symptoms in the subject. The mean ($\pm$ 1 SE) peak rise for norepinephrine at a rate of 10 μg per minute (total dose, 0.150 mg) in 12 subjects was 640 ± 110 μEq per L; and for epinephrine at a rate of 2 μg per minute (total dose, 0.030 mg) in 7 subjects, it was 986 ± 205 μEq per L. These rates of infusion exceeded the minimal amount of both norepinephrine and epinephrine that would produce a rise in plasma FFA level exceeding the "control" response of 150 ± 42 μEq per L (3). For norepinephrine the minimally effective dose was 2 μg per minute (total dose, 0.0075 mg). It is in reference to these initial observations, both as to the magnitude of a peak response and to the minimally effective amount, that the remainder of the compounds were evaluated.

Table I lists the peak response in FFA levels for all compounds studied. Isoproterenol (Isuprel) alone consistently effected a peak response of significant magnitude. It should be noted that many of the compounds failed to result in a significant rise in FFA level when infused in amounts greatly exceeding the effective lipolytic

TABLE I

Peak FFA and blood pressure response to intravenous infusion of catechol compounds and vasoactive polypeptides

Compound	Subjects	Total dose	Peak Δ FFA	Peak B.P. Δ
		mg	*μEq/L*	*mm Hg*
1. Saline	20 subj.	0.780	Mean ± SE: +150 ± 42	+10/+5
2. Norepinephrine	12 subj.	0.150	Mean ± SE: +640 ± 110	+30/+20
3. Epinephrine	7 subj.	0.030	Mean ± SE: + 986 ± 205	+30/+5
4. Isoproterenol	L.C.	0.032	+ 722	+20/−30
	R.J.	0.032	+ 1,726	+40/−30
5. 3,4 Dihydroxy-phenylacetic acid	E.L.	0.324	− 104	−15/−5
6. α-Methyl-dopa	D.C.	450	− 150	−20/−10
7. Dopamine	S.L.	1.060	− 77	−10/−10
	M.H.	7.660	− 364	0/−15
8. 3,4 Dihydroxy mandelic acid	V.C.	0.464	+ 34	−15/−10
9. 3-Methoxy, 4-hydroxy mandelic acid	M.E.	0.364	− 355	−5/0
10. DL-metanephrine	A.McD.	0.324	− 38	0/0
	T.B.	0.648	− 29	0/8
11. Normetanephrine	H.P.	1.620	+ 13	+5/0
12. Ephedrine	J.W.	16	+ 242	+10/+5
	P.B.	16.5	+ 179	+10/0
	P.C.	22.32	+ 122	+12/0
	M.A.	24.00	+ 179	+12/−10
13. Phenylephrine	A.H.	0.780	+ 121	+44/+15
	D.C.	0.540	+ 124	+50/+32
	J.M.	0.540	− 60	+30/+20
14. Metaraminol	V.S.	1.08	+ 117	+30/+15
15. Mephentermine	A.D.	12	+ 323	+10/+5
	L.C.	16	+ 126	+15/+15
	J.F.	16.2	− 54	+20/+10
	T.F.	21.1	+ 70	+40/+20
16. Methoxamine	R.P.	13	− 40	+60/+10
17. Vasopressin	J.J.	1.08μ	+ 95	+5/+5
	A.W.	1.08μ	− 18	+15/+8
18. Angiotensin	H.B.	0.006	− 161	+66/+41
	C.W.	0.017	+ 126	+25/+28
	L.L.	0.017	+ 32	+30/+20
	B.H.	0.017	+ 76	+15/+20
	T.H.	0.017	− 26	+20/+30
	F.B.	0.028	+ 18	+15/+16

or vasoactive dose of norepinephrine or epinephrine.

DISCUSSION

Figure 1 illustrates the presently formulated outline of the biogenesis and degradation of norepinephrine and epinephrine. It was the intent of this study to test as many of the compounds involved in the sequence of chemical events as were available. Of the six compounds actually assayed, neither the two before (dopamine and dopa [1]) nor the four degradative products after (3,4-dihydroxy mandelic acid; 3-methoxy,4-hydroxy mandelic acid; normetanephrine; metanephrine) nor-

[1] α-Methyl-dopa was actually studied, since the addition (rather than any substitution) of a methyl function was not considered inhibiting to a fat mobilizing agent.

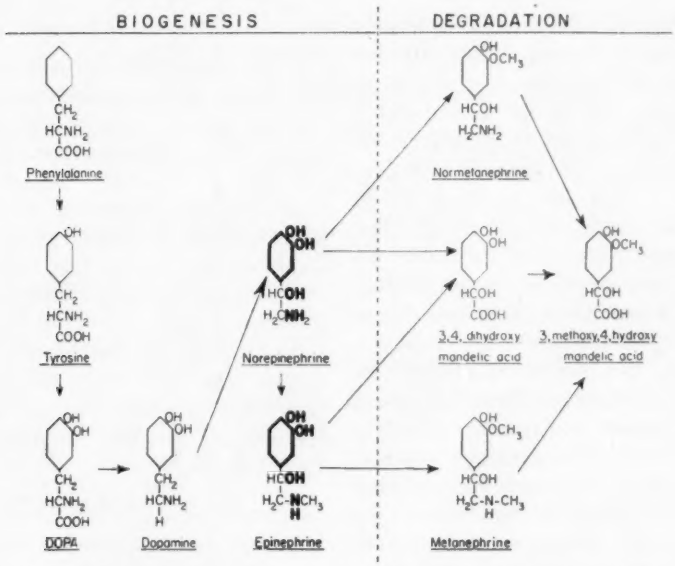

FIG. 1. SCHEME OF BIOGENESIS AND DEGRADATION OF EPINEPHRINE AND NOREPINEPHRINE. Chemical groups in bold outline are the units considered to be necessary for fat-mobilization effect in man.

epinephrine and epinephrine had any significant effect upon the plasma FFA level. These observations led us to believe that the structural configuration of the active compounds may be specific for the fat mobilizing effect, and, accordingly, the series of compounds illustrated in Figure 2 was studied. It will be noted that whenever either or both hydroxyl functions in the phenyl ring

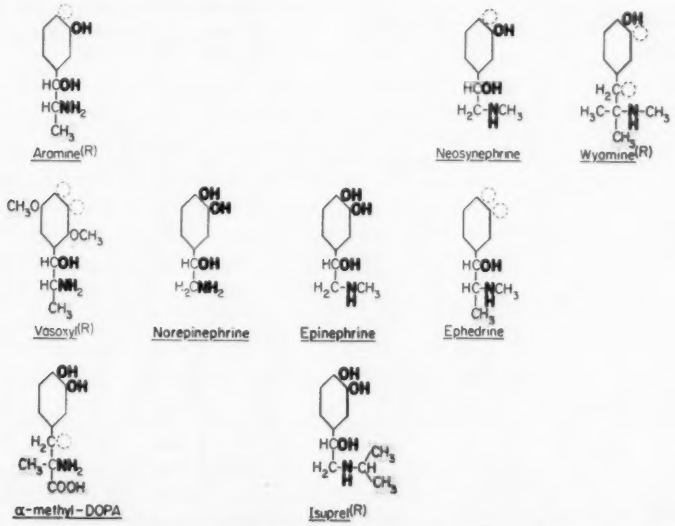

FIG. 2. STRUCTURAL CONFIGURATION OF A SERIES OF COMPOUNDS INFUSED INTO MAN. Chemical groups in bold outline are the units considered to be necessary for fat mobilization in man. Dashed circles indicate absence of a hydroxyl group. Shaded groups indicate addition of a group. See text for FFA response.

were removed [phenylephrine (Neo-synephrine), ephedrine, metaraminol (Aramine), methoxamine (Vasoxyl)] or altered (normetanephrine), FFA effect was lost. The loss of one of the phenyl hydroxyl (3-hydroxy) and the β-carbon hydroxyl group [mephentermine (Wyamine)] also resulted in inactivity. The loss of the β-carbon hydroxyl (dopamine) also modified activity. The addition of two methyl groups to the carbon already bound to the amino nitrogen (isoproterenol) does not modify fat-mobilizing activity. These several structural alterations do not, however, completely remove the vasoactive property of these compounds (Table I). Figures 1 and 2 have been drawn so as to emphasize the four chemical groups which must be present in relationship to the benzene ring to cause a fat-mobilizing response. The FFA activity of isoproternol, containing the four groups, should be noted, because it is a synthetic compound in which the structural alterations have resulted in changes in the specific cardiovascular effect, but in which the fat-mobilizing effect is still present.

The studies of the vasoactive polypeptides were conducted in order to test whether the vascular effects were comparably separable from any lipolytic effects; the absence of any plasma FFA increase would indicate that such an independence of activity does exist.

SUMMARY

1. The effect of the intravenous infusion of a series of catechol compounds and vasoactive polypeptides upon the plasma free fatty acid level of fasting, resting individuals was studied.

2. The presence of a benzene ring with two hydroxyl groups in the 3 and 4 position, a β-carbon hydroxyl and an amino group attached to the α-carbon atom are the four concomitant chemical functions necessary for effective fat mobilizing property in man.

3. Although the polypeptides studied demonstrated vascular activity, no lipid-mobilizing effect was noted, further supporting the suggested specificity of the chemical structure necessary for fat mobilization.

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STUDIES OF THE BLOOD–CEREBROSPINAL FLUID BARRIER TO CORTISOL IN THE DOG *

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This study was undertaken to investigate the entry of administered cortisol and one of its metabolic products into the cerebrospinal fluid (CSF) of dogs under a variety of conditions. Cortisol,[1] one of the principal circulating adrenal cortical steroids of man (1, 2) enters the CSF (3–5), but little is known about the rate at which it passes into the CSF or what factors regulate its passage. Although the relationship of CSF to the extracellular fluid of the brain is complex, it is generally accepted that the two compartments are in equilibrium, and that the blood-CSF and blood-brain barriers do not differ qualitatively in their respective permeability characteristics (6, 7). The data of this study on the entry of cortisol into CSF can therefore be considered relevant to the problem of penetration of cortisol into the brain. This is of some clinical importance with respect to the known effects of cortisol upon brain metabolism; e.g., upon seizure threshold and emotional state (8).

There are abundant data which show that the rate of entry of many substances into the CSF is determined by certain of their physical and chemical properties. These properties are solubility in lipid solvents, binding to plasma protein, molecular size, and in the case of salts of weak acids and bases, pK (6, 7, 9). Since neutral steroids have no pK, and molecular size is not an important determinant of rate of entry into the CSF except with substances of very high molecular weight (6, 7), only lipid solubility and binding to plasma protein (10, 11) would be expected to influence the penetration of cortisol into the CSF. In general, it can be stated that the more lipid-soluble a substance is the more rapidly it enters the CSF, and the less firmly bound it is to plasma protein the higher will be the concentration ratio, CSF/plasma, at equilibrium (6, 7). The purpose of this investigation was to compare the entry into CSF of free cortisol and the water-soluble glucuronides that are important products of cortisol metabolism. The data show that these steroids behave like many other substances (6, 7) in that cortisol, a lipid-soluble compound, penetrates readily into CSF, whereas the water-soluble, lipid-insoluble metabolites, tetrahydrocortisone and tetrahydrocortisol glucuronides, do not.

METHODS AND MATERIALS

Thirty-five experiments were carried out in 16 mongrel dogs ranging in weight from 9 to 22 kg. The animals were anesthetized with intravenously administered pentobarbital sodium in a dose of 30 mg per kg body weight. Sufficient additional sodium pentobarbital was given intravenously during the experiments to maintain a stable respiratory rate. Cisternal punctures were performed with a 20-gauge spinal puncture needle which was left *in situ* during each experiment. Whenever CSF so obtained was bloody or xanthochromic, the experiment was terminated and the results were discarded.

Clear CSF, in samples of 0.8 to 1.0 ml, were removed at intervals and, in some instances when detailed chemical studies of the steroids in CSF were planned, amounts as large as 15 ml were withdrawn by allowing the open needle to drain for periods of 30 to 60 minutes. Specimens of CSF were frozen within 1 hour after withdrawal and kept at −20° C until analyzed for steroid content.

Samples of blood (5 to 20 ml) were withdrawn in heparinized syringes from occluded veins of fore- or hindlegs. Plasma was quickly separated from cells and stored at −20° C. In five instances urine specimens were obtained by catheter from female dogs.

The principal experimental procedure was the intravenous injection over a period of 30 seconds of cortisol

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† John and Mary R. Markle Scholar in Medical Science.

[1] The following trivial names for steroids are used in this paper: cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; tetrahydrocortisone, 3α,17α,21-trihydroxy-pregnane-11,20-dione; tetrahydrocortisol, 3α,11β,17α,21-tetrahydroxy-pregnane-20-one; *allo*-tetrahydrocortisol, 3α,11β,17α,21-tetrahydroxy-*allo*pregnane-20-one.

in the form of its water-soluble ester, the 21-hemisuccinate.[2] The dose was 8 mg of cortisol (calculated as milligrams of the free steroid) per kg body weight. The amount chosen was large *1)* in a deliberate attempt to achieve high, readily measurable concentrations of cortisol, *2)* in order to approximate pharmacologic doses, and *3)* in order to achieve high plasma concentrations of glucuronides of cortisol metabolites so as to afford the best possible opportunity for these glucuronides to penetrate the blood-CSF barrier. Samples of blood were then drawn at 5 minutes, 30 minutes, and at 30- to 60-minute intervals thereafter. In 6 animals this procedure was carried out before and after 11 days of treatment with intramuscular corticotropin, 40 IU per day.[3] When cortisol alcohol or one of its principal metabolites, tetrahydrocortisone, was injected, the steroid was dissolved in small amounts (5 to 10 ml) of 95 per cent ethanol and the solution was dissolved in 50 to 100 ml of isotonic saline for rapid intravenous infusion.

In 4 animals additional experiments were done in order to detect the specific steroids appearing in blood after cortisol infusion. In these studies, cortisol (as the alcohol, not the 21-hemisuccinate) was administered as described, and large volumes of blood were withdrawn after 1 to 3 hours and the plasma separated and stored at $-20°$ C.

Determinations of 17-hydroxycorticosteroids (cortisol) in plasma, CSF and urine were made by the Silber-Porter technic (12) as modified in this laboratory (13) and by Peterson, Karrer and Guerra (14). In all instances optical densities of the cortisol-phenylhydrazine reaction product were measured at several wave lengths. To insure specificity in the cortisol determination, all samples not showing an absorption maximum at 410 mμ (the absorption maximum of cortisol in this reaction) were discarded. Cortisol measured by this technic is termed "free" steroid, in the sense that it is unconjugated—i.e., not in the form of metabolites reduced in the A ring and conjugated at the 3 position with glucuronic acid. To obtain information about the concentration of such glucuronides, more detailed studies were done. From large volumes of plasma, CSF, and urine, obtained after administration of cortisol, "free" cortisol was partially identified after ethyl acetate extraction, by paper chromatography with an authentic cortisol standard in Bush systems (15), by ultraviolet light absorption at 240 mμ, by reaction with blue tetrazolium, and measured by the Silber-Porter reaction. For detection of the glucuronides of cortisol metabolites, the aqueous phase, after ethyl acetate extraction for free cortisol, was subjected to hydrolysis. In the case of plasma, protein was first precipitated with large volumes of ethanol, the ethanol was evaporated, the aqueous residue was then diluted with distilled water, and the mixture hydrolyzed. For plasma, CSF, and urine, the hydrolytic procedure was the same. β-glucuronidase (beef liver) was added to the aqueous phase in concentrations of 300 to 500 units per ml. The pH was kept at 4.5 to 5.0 with 0.1 sodium acetate-acetic acid buffer. The mixture was incubated for 48 to 120 hours at 37° C. Hydrolysates were then extracted with ethyl acetate, and the steroids released were chromatographically located and characterized, and measured as above. Recovery of cortisol added to plasma, CSF, and urine is quantitative (85 to 105 per cent). With the hydrolytic procedure used, there is no inhibition of the action of β-glucuronidase upon steroid glucuronides by canine CSF (16).

[2] Courtesy of the Upjohn Co.

[3] Courtesy of Organon, Inc.

RESULTS

1. Resting levels of cortisol in plasma and CSF of dogs. Thirteen measurements in 11 anesthetized dogs yielded an average value for plasma 17-hydroxycorticosteroid concentration of 10 μg per 100 ml, a value in the same range as that reported for dogs by Eik-Nes and Brizzee (Table I) (17). The range of values was wide—0 to 33 μg per 100 ml. In the same 11 dogs, measurements carried out on 1-ml samples of CSF revealed no detectable cortisol. The validity of a virtually zero value for cortisol in CSF was confirmed by attempts to detect cortisol in CSF samples as large as 7 ml, a volume in which the method is sensitive enough to measure reliably concentrations of less than 1 μg per 100 ml. Chromatographic studies of similar volumes of CSF also failed to reveal the presence of cortisol.

2. Entry of infused cortisol into CSF. Cortisol-21-hemisuccinate, 8 mg per kg, was rapidly injected intravenously in 18 experiments upon 14 anesthetized dogs. In 11 experiments, samples of CSF were obtained 5 minutes after cortisol injection, and in 3 instances detectable concentrations of cortisol were found, indicating rapid entry of the steroid into the CSF compartment (Table I). In all but one experiment cortisol appeared in the CSF 30 minutes after injection. Figure 1, in a typical experiment, shows entry of cortisol into the CSF 5 minutes after administration and a rising concentration of cortisol in CSF, reaching a maximum at 90 minutes, while the concentration of plasma cortisol was falling. The figure also indicates the high plasma cortisol concentration at 5 minutes. In the 10 dogs in which plasma cortisol levels were determined at 5 minutes, the range of concentrations was 447 to 1,440 μg per 100 ml. (The wide range of values can be attributed in part to errors of 30 to 60 seconds in obtaining the 5-minute sample.) These high values constitute

TABLE I

Concentration of cortisol in plasma and CSF of 11 dogs after rapid intravenous injection of 8 mg of steroid (cortisol 21-hemisuccinate) per kg body weight

Dog	Minutes:	17-Hydroxycorticosteroids (cortisol), μg %								
		0	5	30	60	90	150	180	210	240
WN	Plasma	0		535	275	171	158		81	
	CSF	0		21	44	26				
TA	Plasma	0	447	350		168		104		
	CSF		0	62		71		45		
WH	Plasma	0	1,350	353		159		61		42
	CSF	0	0	48		42		15		0
BR	Plasma	17	1,221	574	433	268	148			
	CSF	0	16	68	68	78	45			
CH	Plasma	0	816	534	299	210	150		109	
	CSF	0	0	20	52	61	42		22	
TO	Plasma	5	875	273	163	111	79			
	CSF	0	0	71	90	61	38		0	
SH	Plasma	33	1,030	505	296	230	141		94	
	CSF	0	4	61	85	71	40			
DA	1) Plasma	21	880	382	244	173	88			
	CSF	0	0	54	65	73	43			
	2) Plasma	20	1,120	365	230	195	73			
	CSF		0	64	47	45	34			
PY	1) Plasma	16	810	313	178	149	77			
	CSF	0		0	28	23	17			
	2) Plasma	9	822	376	230	144	78			
	CSF	0	11	32	31	38	23			
LE	Plasma	3	1,440	480	254	177	114			
	CSF	0	0	76	94	72	54			
RA	Plamsa	2	1,173	372	207	145	76			
	CSF	0	0	41	87	94	54			

evidence that the injected material, cortisol hemisuccinate, measured only to a limited extent by the Silber-Porter reaction is rapidly hydrolyzed *in vivo* to free cortisol which is measured by this colorimetric test.[4] The graph illustrates a constant finding in all studies, namely that in short-term studies (90 to 120 minutes in duration) with a rapidly falling plasma cortisol concentration, levels of cortisol in CSF never equaled those in plasma. Maximal concentration of cortisol in CSF was reached 60 to 90 minutes after infusion of the steroid.

The rate of disappearance of cortisol was rapid. In the 18 experiments the range of plasma "half-time" was 40 to 93 minutes, with an average of 64 minutes. In six animals the rate of disappearance from plasma of cortisol was calculated in terms of the slope obtained by the method of least squares. This calculation was done in only those

[4] Cortisol-21-hemisuccinate, added *in vitro* to Addisonian plasma with a zero level of cortisol, has a chromogenicity at 410 mμ in the Silber-Porter reaction equal to about 15% of the chromogenicity of free cortisol itself. Therefore, if the steroid ester were incompletely hydrolyzed *in vivo*, which cannot be excluded in the early (5-minute) samples, the Silber-Porter technic would overestimate plasma cortisol level to a small degree. For example, if as much as 50% of the ester were not hydrolyzed (and it is unlikely that this much escapes hydrolysis), the concentration of free plasma cortisol would be overestimated by 7.5% (0.50×0.15). If 20% remained unhydrolyzed, the overestimation would be 3% (0.2×0.15). From 30 minutes on it seems unlikely that any of the steroid ester remains unhydrolyzed. There are data indicating that pharmacologic doses of cortisol hemisuccinate behave in general like free cortisol, both in terms of metabolic fate and absolute values of plasma cortisol attained (18). As will be seen (Section 3 below), the same distribution between plasma and CSF is found with cortisol-21-hemisuccinate as with free cortisol.

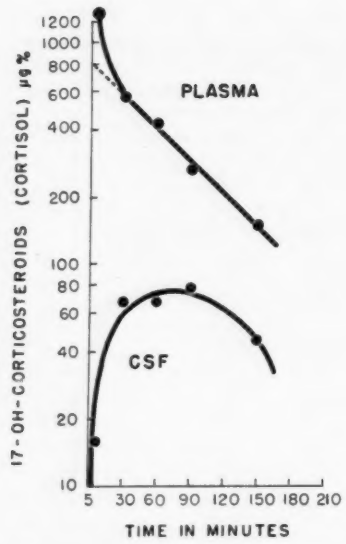

FIG. 1. PLASMA AND CSF CONCENTRATIONS OF CORTISOL IN DOG BR (SEE TABLE I) AFTER RAPID INTRAVENOUS INJECTION OF CORTISOL 21-HEMISUCCINATE, 8 MG PER KG. Concentration in plasma is high 5 minutes after injection and the steroid is detectable in CSF at that time.

instances in which there was a sufficient number of determinations for adequate mathematical analysis (Table II). The decrease in plasma cortisol level averaged 47.3 per cent per hour, with a range of 40.2 to 53.7. This rate of disappearance

TABLE II

Rate of disappearance of cortisol after administration of cortisol 21-hemisuccinate from plasma of 6 dogs before and after corticotropin administration for 11 days *

Dog	Rate of cortisol disappearance	
	Before corticotropin	After corticotropin
	% decrease/hr	
TA	40.2	40.8
BR	50.0	45.6
CH	45.5	34.3
TO	42.4	48.9
LE	50.5	57.3
RA	53.7	50.2
Mean	47.3	46.7
SD	5.22	7.98

* The data are derived from calculation of the slope of the straight line describing the logarithmic decrement of plasma cortisol concentration. Slope is calculated by the method of least squares. The disappearance rates are essentially the same before and after corticotropin ($p > 0.50$).

is considerably more rapid than that found in normal human subjects (27 per cent per hour) (18) or "half-time" of 138 minutes (19).

3. The steady state distribution of cortisol between plasma and CSF. A constant infusion of cortisol 21-hemisuccinate, 300 mg, was given in 1,500 ml 5 per cent D/W over a 9-hour period to a 15 kg dog after a priming dose of 22.5 mg. Samples of plasma and CSF were obtained after 4 hours and maintained for the succeeding 5 hours. Cortisol concentration in plasma during this equilibrium state was 200 µg per 100 ml; in CSF, 40 µg per 100 ml. The distribution ratio CSF/plasma of 0.2 is not corrected for protein-binding of the steroid (10, 11) nor for the differences in water content of the two compartments.

A second steady state experiment in another animal was done with cortisol alcohol. At equilibrium the concentration ratio, CSF/plasma of cortisol (53/211 µg per 100 ml) was 0.25. The similarity of the CSF/plasma ratios in the two studies is interpreted to signify that the steroid hemisuccinate behaves physiologically like the free steroid.

4. Identification and measurement of steroid entering the CSF. In this series of experiments an attempt was made to learn whether or not certain water-soluble metabolites of cortisol, the A-ring reduced, 3-glucuronides, entered the CSF after cortisol injection. These metabolic products are among the chief urinary steroids recovered in urine after administration of cortisol (20). Accordingly, these reduced steroids were sought in blood and urine as well as in CSF (see Methods). Fourteen experiments were carried out in this effort to find tetrahydrocortisone or tetrahydrocortisol in CSF. In five instances measurements for tetrahydrocortisol glucuronide were made in serial small samples (1 ml) of CSF after cortisol hemisuccinate injection, at times when CSF levels of free cortisol were elevated. In no case was tetrahydrocortisol glucuronide found. In nine further studies after cortisol injection, the detailed measurements were made in blood and urine to ascertain that plasma concentrations of tetrahydrocortisol glucuronide were high enough to allow passage into the CSF. After injection of cortisol alcohol, the steroid was recovered from all the fluids investigated; 200 mg of cortisol was given intravenously, and blood was collected during the

period, 30 to 100 minutes after injection, while CSF was being drained through an indwelling cisternal needle. In a representative experiment the plasma level of free cortisol was 1,040 μg per 100 ml, while the level in CSF was 183. Glucuronide content, measured as Silber-Porter reactive material after glucuronidase hydrolysis, was 219 μg per 100 ml in plasma—a high value—while none was detectable in CSF.

The apparent failure of steroid glucuronides to enter CSF was further studied. In four dogs steroids were isolated from blood, CSF, and urine by paper chromatography (see Methods). In all instances cortisol was identified in the three fluids after steroid injection (100 to 300 mg of free cortisol in two instances, of cortisol 21-hemisuccinate in the remaining two). Tetrahydrocortisol glucuronide was identified in blood and urine, never in the CSF. [It is of considerable interest from the point of view of steroid metabolism that no tetrahydrocortisone was recovered after cortisol injection. It appears that this represents a species difference between dog and man; in man, cortisol is metabolized to tetrahydrocortisol, *allo*-tetrahydrocortisol, and tetrahydrocortisone (21).] Pooled serial blood samples from the four animals showed an average value for free cortisol after chromatography of 180 μg per 100 ml. The mean value of plasma tetrahydrocortisol after hydrolysis and chromatography was 70 μg per 100 ml. The two steroids were also indentified in large quantities in the urine. In the 4-hour urine aliquot it was possible to recover 12.5 mg of conjugated tetrahydrocortisol, 7.7 mg of free cortisol. Chromatographic analysis of many large samples of CSF yielded an average cortisol value for the four dogs of 109 μg per 100 ml. These studies failed to reveal even trace amounts of tetrahydrocortisol after glucuronidase hydrolysis.

The data indicate that cortisol is metabolized to a reduced steroid which is conjugated with glucuronic acid, but that this metabolite, tetrahydrocortisol glucuronide, does not detectably enter the CSF, although present in plasma in high concentration.

5. Entry into CSF of tetrahydrocortisone, an A-ring-reduced, unconjugated metabolite of cortisol. The possibility existed that some property of tetrahydrocortisol other than its existence in plasma as a water-soluble conjugate of glucuronic acid was responsible for its exclusion from CSF. A closely related compound, free tetrahydrocortisone,[5] 8 mg per kg body weight, was given intravenously over 30 seconds to two dogs. The free steroid entered the CSF rapidly, as is shown in Figure 2 and Table III. In the experiment illustrated, concentration of 17-hydroxycorticosteroid in CSF at 5 minutes was 66 μg per 100 ml (Table III). These concentrations of steroid in CSF were higher than those attained in any of 13 experiments with cortisol at the 5-minute point (Table I). After cortisol injection, concentrations of the steroid in CSF at 5 minutes ranged from 0 to 16 μg per 100 ml in the face of concentrations in

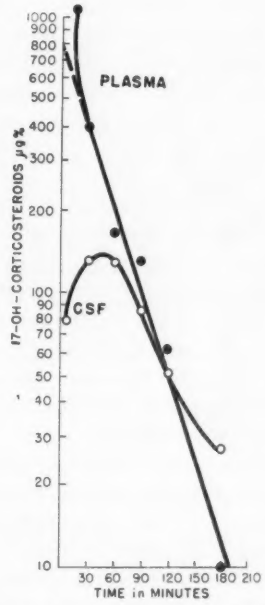

FIG. 2. PLASMA AND CSF CONCENTRATIONS OF TETRAHYDROCORTISONE IN DOG BL AFTER RAPID INTRAVENOUS INJECTION OF TETRAHYDROCORTISONE, 8 MG PER KG. Concentration of steroid in CSF at 5 and 30 minutes is higher than concentration attained after comparable doses of cortisol (see text). Rate of tetrahydrocortisone disappearance from plasma is nearly twice as rapid as that of cortisol (rate of decrease of plasma level, 74% per hour, or plasma "half-time," 35 minutes).

[5] Provided through the courtesy of Merck and Co. Tetrahydrocortisone was used instead of tetrahydrocortisol because the latter steroid was not readily available in large quantities. Since tetrahydrocortisone and tetrahydrocortisol behave alike physiologically (22), it is reasonable to assume that they are interchangeable for the purpose of this experiment.

TABLE III

Concentration of tetrahydrocortisone in plasma and CSF of Dogs BL and DA after rapid intravenous injection of 8 mg of steroid per kg body weight *

Dog	Minutes:	17-Hydroxycorticosteroids (tetrahydrocortisone), μg % 5	30	60	90	120	150	180	240
BL	Plasma	1,090	412	166	130	62		8	0
	CSF	82	133	133	87	51		27	0
DA	Plasma	955	391	219	125	77	65		
	CSF	66	48	46	43	29	26		

* The data show the entry of large amounts of tetrahydrocortisone into the CSF 5 minutes after injection of the steroid.

plasma comparable with those found after tetrahydrocortisone injection. In the two experiments the rate of disappearance of tetrahydrocortisone was rapid, plasma "half-time" being 35 and 39 minutes. Calculated from the slope derived by the method of least squares, decrease in plasma level was 74 per cent per hour, compared with a mean decrease of 47.3 per cent per hour for cortisol (Table II). Free tetrahydrocortisone and tetrahydrocortisone glucuronide were identified in plasma and urine, only free tetrahydrocortisone in the CSF. The more rapid rate of disappearance of tetrahydrocortisone than of cortisol (23), and the virtual absence of conversion of tetrahydrocortisone to tetrahydrocortisol in the dog, are findings in agreement with those made in human subjects (24).

6. The effect of adrenocorticotropin upon the permeability of the blood-CSF barrier to cortisol. This section summarizes the results of determination of cortisol clearance rate and entry into CSF before and after 11-day courses of corticotropin in six dogs. ACTH administration had no effect upon the rate of clearance of cortisol from plasma. Table II shows that the slope of the line describing cortisol disappearance rate is unchanged after 11 days of corticotropin. The rate of cortisol disappearance in the control studies averaged 47.3 per cent per hour; the average after ACTH administration was 46.7 per cent per hour, $p > 0.5$.

Analysis of ratios of CSF/plasma concentrations of cortisol at 30, 60, 90 and 150 minutes showed no significant difference between the control and experimental values. This schedule of corticotropin administration therefore does not appear to alter the permeability of the blood-CSF membranes with regard to cortisol.

Comparison of the disappearance rates and cortisol entry into CSF at different times (i.e., 11 days apart) seems a valid procedure in view of a control study in which three dogs received standard cortisol-21-hemisuccinate infusions before and after periods of 11 days during which no ACTH was given. None of the indices changed significantly during this time.

DISCUSSION

The data show that free cortisol enters the CSF rapidly in the dog after intravenous injections of large amounts of the steroid. The failure to detect cortisol in CSF of the "resting" dog is in agreement with the data Abelson, Baron and Toakley obtained from human subjects (4). They reported low cortisol concentrations in human CSF (0.2 to 0.4 μg per 100 ml) in the resting state, using very large volumes of CSF for chromatographic isolation and quantitation of cortisol (4). Sandberg, Eik-Nes, Nelson and Tyler (3) found no detectable cortisol in small samples of "resting" CSF in man, except for ten instances of central nervous system disease in which the permeability of the blood-CSF barrier is presumably altered (acute poliomyelitis, pneumococcal meningitis, cerebral vascular accidents). In man, Abelson, Baron and Toakley (4) and Cranny and Kelley (5) have demonstrated the rapid entry of cortisol into CSF after administration of cortisol or cortisone, and Cranny and Kelley presented evidence suggesting that the amount of cortisol entering the CSF is increased by the presence of meningeal infection (5). The single observation of Hellman and associates (25) that cortisol-4-C^{14} could not be found in human CSF 8 hours after administration of the steroid can probably be ascribed to the fact that the CSF level after 8 hours would be expected to be exceedingly low.

The data of the present report also show that after injection of cortisol in the dog, only free cortisol, not its reduced glucuronide conjugates, enters the CSF in measurable quantities over periods of 30 to 210 minutes. The rate of entry of steroid, as of other substances (6, 7, 9), into the CSF is dependent upon several factors. Among these are the permeability characteristics of the complex membranes which constitute the blood-CSF "barrier." These include the membranes of the cerebral capillary endothelium, the pia-glial

complex, the cerebral astroglia, choroid plexus, and the ependymal and arachnoidal surfaces (6, 7). The blood-CSF barrier serves to stabilize the composition of the CSF and the extracellular fluid of the brain, and contributes to the maintenance of a constant internal environment for the central nervous system.

The entry of cortisol into the CSF, and the exclusion from the CSF of water-soluble steroid glucuronides are findings in agreement with those made in the case of other natural and synthetic compounds (6). It appears that a major factor, perhaps the chief factor that determines the difference in penetration into the CSF between free cortisol and tetrahydrocortisone, on the one hand, and tetrahydrocortisol glucuronide and tetrahydrocortisone glucuronide on the other, is the high degree of lipid-solubility of the free (or undissociated) form and the negligible lipid solubility of the polar, dissociable glucuronide. Since no steroid glucuronide entered the CSF when plasma concentrations were far above the physiologic range, it is hardly conceivable that they cross the blood-CSF barrier at the lower concentrations obtaining under physiologic conditions.

The influence of lipid-solubility upon the rate and degree of entry into CSF (steady state CSF/plasma ratio) of such foreign substances as sulfonamides and barbiturates is marked: the greater the lipid-solubility of the particular substance, the greater is its penetration into the CSF (6). To date, no such orderly arrangement can be made in the case of the free steroids as a group, because only cortisol has been studied extensively.

In addition to lipid-solubility, molecular size might be supposed to influence entry of compounds into CSF (6, 26). However, the relatively small difference in molecular weight between cortisol (mol wet 360) and tetrahydrocortisol glucuronide (mol wt 540) is considered to be of negligible importance in view of the rapid entry of molecules as large as serum albumin into the CSF (27).

The pK of a compound plays a role in the degree and rate of its entry into CSF: pK influences the plasma-CSF distribution of salts of weak acids and bases (9). The normally lower pH of CSF as compared with that of plasma does not influence the distribution of non-ionized substances such as free steroid hormones. The pH gradient would tend to favor the exclusion of weak acids such as the glucuronides of steroids (9). There are no available data concerning the pK of steroid glucuronides; one would expect it to approximate that of glucuronic acid itself.

The degree to which a substance is bound to protein is also of great importance in determining its distribution (steady state ratio) between plasma and CSF. This is true of calcium, bilirubin and many drugs (6). The observed steady state ratio (CSF/plasma) of cortisol of approximately 0.2 may be considered a consequence of binding of cortisol to plasma protein. There is disagreement in the literature concerning the precise quantity of cortisol that is physiologically bound to protein in man. The estimates range from 72 to 94 per cent bound to protein at 37° C, the per cent bound being in part dependent upon the plasma concentration of cortisol (11, 22, 28–30). There is also disagreement about the degree of cortisol binding to plasma protein in the dog. Daughaday found little evidence of corticosteroid-binding-globulin activity in canine plasma, and ascribed most of the 60 to 75 per cent binding observed to albumin (30). Slaunwhite and Sandberg found that only 8 to 40 per cent of added cortisol was bound by dog plasma (11), but Florini and Buyske reported that cortisol binding to plasma in the dog is similar to that in man—i.e., 60 to 90 per cent over a wide range of concentrations (31). The present study provides no data which resolve these differences. However, the observed steady state ratio of 0.2 (CSF/plasma) is compatible with the estimate that 20 per cent of cortisol is unbound, and available for entry from plasma into CSF.[6]

A transfer rate constant (9) for cortisol was not derived because almost all of the data were obtained after a single, rapid intravenous injection of cortisol rather than with maintenance of a constant plasma level. This experimental design was chosen primarily to obtain information regarding the disappearance of cortisol from

[6] The data are comparable with those obtained by Upton and Bondy who studied cortisol binding to plasma protein in human subjects (32). Over a wide range of high concentrations of cortisol in human plasma, these workers found that about 15% of the steroid was ultracentrifugable, i.e., that 85% was tightly bound to plasma protein (presumably to the globulin fraction), and 15% was bound only loosely (perhaps to albumin) (10, 32).

plasma which could not be readily obtained from steady state experiments.

The data show clearly that free tetrahydrocortisone disappears from plasma and enters CSF more rapidly than does free cortisol. This difference may be explained in part by the fact that less tetrahydrocortisone than cortisol is bound to plasma protein (33). At present, data concerning tetrahydrocortisone binding to plasma protein have been reported only in man (33), and it may not be permissible to assume a similar difference in binding of the two steroids in dog plasma. However, no other physical or chemical property appears to account so well for the differences noted, the lipid solubilities and molecular weights of the two steroids being nearly identical.

The high levels of cortisol observed in CSF after injection of large amounts of the steroid are considered to approximate the "diffusible" fraction of the steroid in the plasma. The high concentrations of hormone in the CSF achieved by parenteral administration lead to the conclusion that intrathecal administration of steroids is unnecessary. If one assumes that cortisol is distributed like other lipid-soluble substances, then the concentration of the steroid at equilibrium would be higher in brain than in either CSF or plasma (6). In any disease process that is associated with an increased concentration of protein in the CSF, reflecting a change in the permeability characteristics of the blood-CSF membranes, one would anticipate that concentrations of cortisol in CSF after oral or intravenous administration would be considerably higher than those observed under the relatively normal conditions of the studies here reported.

The data of the present study appear to show that intramuscular administration of ACTH for 11 days does not enhance the penetration of cortisol into the CSF. The same dosage schedule of ACTH has also been shown to bring about no change in the exchange of sodium (Na^{24}) between plasma and CSF (34). The absence of an altered rate of cortisol clearance from plasma after ACTH administration is a finding in accord with certain earlier studies, but not in agreement with others. Berliner, Keller and Dougherty (35) demonstrated in mice that pretreatment with corticotropin did not prolong the plasma half-time of injected cortisol. Wallace, Leilop, Carter and Lyons (36) found no decrease in rate of cortisol disappearance from plasma in human subjects treated with intramuscularly administered ACTH for long periods. In contrast, Kuipers, Ely and Kelley (37) observed a modest prolongation of plasma half-time of infused cortisol after the acute intravenous injection of corticotropin. Dougherty and Berliner (38) reported similar findings in mice, and DeMoor, Hendrikx and Hinnekens reported a delay in disappearance of cortisol from plasma in normal and Addisonian human subjects immediately after ACTH administration (39). Berliner and co-workers have attempted to explain such a prolongation of plasma half-time of cortisol by their reported observations of inhibition by ACTH of hepatic conjugation of metabolites of this steroid with glucuronic acid in rats and mice, an "extra-adrenal effect" of ACTH (35). This hypothesis remains to be proved. In the meantime, the differences in results may be best accounted for by differences in route and chronicity of ACTH administration.

SUMMARY

1. The resting concentration of cortisol in cerebrospinal fluid (CSF) is less than 1 μg per 100 ml in the anesthetized dog.

2. After intravenous injection of cortisol or cortisol-21-hemisuccinate, the steroid enters the CSF rapidly, appearing within 5 to 30 minutes.

3. In a single experiment prolonged infusion of cortisol-21-hemisuccinate resulted by the fourth hour in the attainment of a steady state ratio, CSF/plasma, of 0.2. This can be interpreted as reflecting an 80 per cent binding of cortisol to plasma protein.

4. After cortisol or cortisol-21-hemisuccinate injection, only free (unconjugated) steroid, not the glucuronides of cortisol metabolites, appears in CSF, although a conjugated metabolic product, tetrahydrocortisol glucuronide, is found in high concentration in plasma and urine. These findings are in accord with those concerning most drugs and natural products; i.e., that nonpolar, water-insoluble substances enter CSF from blood more readily than do polar, water-soluble compounds.

5. Another metabolic product of cortisol, tetrahydrocortisone, freely enters the CSF, but tetrahydrocortisone glucuronide does not. The more

rapid entry into CSF of tetrahydrocortisone than of cortisol may be explained by the lesser degree of binding to plasma protein of the former steroid.

6. In the dog, cortisol appears to be metabolized to tetrahydrocortisol only, not to tetrahydrocortisol, *allo*-tetrahydrocortisol, and tetrahydrocortisone, as in man.[7]

7. Administration of corticotropin for 11 days to 6 dogs did not enhance the entry of administered cortisol into the CSF, nor did it alter the rate of clearance of cortisol from plasma.

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STUDIES ON COPPER METABOLISM. XXXI. ERYTHROCYTE COPPER *

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The isolation and characterization of a cuproprotein, erythrocuprein, from normal human erythrocytes have been described recently (1–3). Preliminary studies indicated that normal human erythrocytes contain about 30 to 36 mg of erythrocuprein per 100 ml of packed cells (1). Since erythrocytes are known to hold about 100 μg of copper per 100 ml of packed cells (4), and since erythrocuprein carries 3.4 μg of copper per mg of protein, we suggested that erythrocuprein accounts for most of the copper in erythrocytes.

It became apparent in the course of further investigation that the anti-erythrocuprein serum used in the preliminary studies contained antibody to an erythrocyte component other than erythrocuprein and that the erythrocuprein concentrations reported were erroneously high. A modified technic for purification of erythrocuprein, as well as immunological evidence for the homogeneity of the purified product, are presented in this report. We shall also report studies of erythrocuprein in normal human erythrocytes and in those from patients with various diseases. Since erythrocuprein accounted for only about 60 per cent of the erythrocyte copper, observations are included on the preliminary separation of an additional erythrocyte copper component(s).

METHODS

Preparation of erythrocuprein. Erythrocuprein was prepared from normal human erythrocytes by a modification of the method described previously (1). The substances recovered from the lead acetate precipitation (step 3) were dialyzed free of phosphate ion and stored as a lyophilized powder (LP); 1 to 2 g of LP was chromatographed (Figure 1A) on a 5 cm (47 mm internal diameter) × 40 cm column containing 100 g diethylaminoethyl (DEAE)-cellulose, prepared by the method of Peterson and Sober (5). A linear phosphate concentration gradient with a constant pH of 6.8 was used. The erythrocuprein-containing fractions were identified by the high copper to nitrogen ratio and the reaction with specific antiserum in the agar diffusion technic, as described below. The fractions containing erythrocuprein from several chromatograms were then pooled and rechromatographed (Figure 1B) under conditions identical with those used in the first chromatogram.

The erythrocuprein obtained was homogeneous in the ultracentrifuge. The molecular weight of the protein, determined by the Archibald approach to equilibrium as modified by Schachman (6), was 31,000 ± 6 per cent. The copper (μg) to nitrogen (mg) ratio was 23.0.

Preparation of antiserum. Rabbits were immunized at weekly intervals for 4 weeks. After a rest period of 1 month a second course of four weekly injections was given. Each subcutaneous injection contained erythrocuprein (0.25 mg) or LP (20 mg) dissolved in 0.5 ml of 0.85 per cent sodium chloride, 0.5 ml of 15 per cent Arlacel A (vol/vol) in Bayol F,[1] and 1 mg of heat-killed tubercle bacilli. After immunization was complete the serums from several rabbits were pooled and merthiolate was added to the serum to a final concentration of 1:10,000. The pooled antiserum was stored at 5° C.

Evidence for the immunologic homogeneity of anti-erythrocuprein serum. The agar double diffusion technic of Halbert, Swick and Sonn (7) was used as a criterion of immunologic homogeneity of both the purified erythrocuprein and the anti-erythrocuprein serum. Three-tenths ml of either antiserum (undiluted or diluted 1:1) or antigen (1 mg per ml in saline) was pipetted into each well. The agar plates were incubated at room temperature and observed at approximately 12-hour intervals for 3 to 12 days.

Erythrocuprein (E-I), prepared as described in the previous publication (1), and antiserum (AS-I) to this batch of erythrocuprein were available for study. These preparations, as well as erythrocuprein purified chromatographically (E-II), were tested by the agar double diffusion technic. Typical results are presented diagrammatically in Figure 2.

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[1] Arlacel A (mannide monooleate), Atlas Powder Co.; Bayol F, Penola Oil Co.

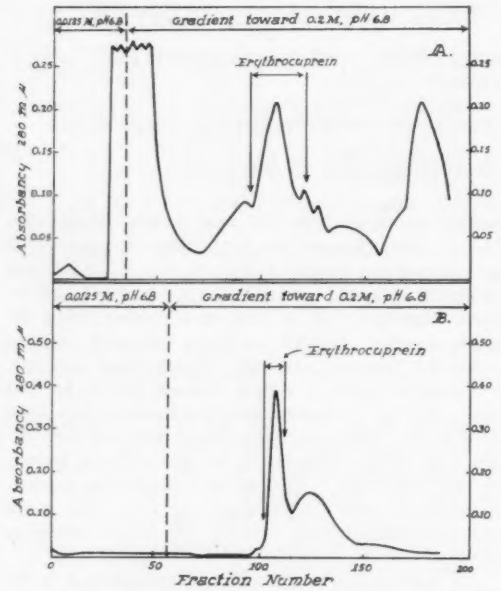

FIG. 1. CHROMATOGRAMS OF AN EXTRACT (LP) PREPARED FROM A HEMOLYSATE OF NORMAL HUMAN ERYTHROCYTES. The lyophilized powder containing erythrocuprein was dissolved in 0.0125 M phosphate buffer and chromatographed on DEAE-cellulose. The fractions containing erythrocuprein (A) were pooled and rechromatographed (B) in order to obtain highly purified erythrocuprein.

When E-I was compared in the agar-diffusion procedure with a hemolysate prepared from a normal adult subject (H-A) by the use of AS-I, a single band developed (Figure 2a) which exhibited a reaction of identity (8). When hemolysates prepared from 12 samples of normal umbilical vein blood (H-F) were tested in the same manner, reactions suggesting a contaminating antigen were observed (Figure 2, b and c). Therefore, in an effort to confirm the presence of a contaminating antigen, various fractions obtained by chromatography (Figure 1A) of the lyophilized powder (LP) were tested with AS-I. The contaminating antigen, referred to as CA, was localized in the initial effluent from the column (0.0125 M phosphate buffer, pH 6.8) and was entirely free of copper. When CA was tested with AS-I, a single band was observed which gave a reaction of partial identity with E-I (Figure 2d). Erythrocuprein (E-I), to which CA had been added, reacted with AS-I to form two bands (Figure 2e), indicating that the small amount of CA in the erythrocuprein (E-I) was not resolved in most instances by the agar diffusion technic.

Serial absorption of AS-I with CA resulted in an antiserum that no longer reacted with CA (Figure 2f). This absorbed antiserum (AS-I absCA) gave only one precipitin band when tested against E-I and H-F (Figure 2g).

Antiserum prepared from E-II (AS-II) reacted with E-II but not with CA (Figure 2h) and the reaction with E-II was identical with that between AS-II and E-I (Figure 2i). Only one band was observed when AS-II was used to compare E-II with H-A, H-F, and LP (Figure 2, j, k, and l).

Thus, E-I contained a small amount of a highly antigenic impurity (CA). This could be demonstrated in the agar double diffusion technic only by enriching the erythrocuprein preparation with the contaminant. Erythrocuprein preparations that were purified by chromatography on DEAE-cellulose were free of this impurity. Antiserum (AS-II) prepared from the highly purified erythrocuprein (E-II) was used thereafter for the measurement of erythrocuprein.

Measurement of erythrocyte copper and erythrocuprein. Suspensions of human erythrocytes from which the buffy coat had been removed were washed twice with cold 0.85 per cent sodium chloride. The cells were suspended in saline and the volume of packed red cells (VPRC) was determined. Aliquots of the suspension (3.0 ml) were taken in duplicate for the determination of eryth-

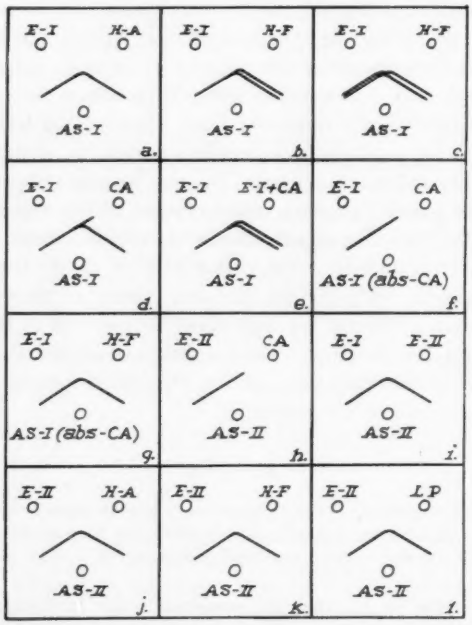

FIG. 2. DIAGRAMMATIC REPRESENTATION OF AGAR DOUBLE DIFFUSION PLATES. E-I, erythrocuprein prepared as described previously (1); E-II, erythrocuprein purified chromatographically; AS-I, antiserum to E-I; AS-II, antiserum to E-II; H-A, hemolysate of normal adult erythrocytes; H-F, hemolysate of umbilical vein erythrocytes; CA, contaminating antigen; AS-I absCA, AS-I absorbed with the contaminating antigen (CA); LP, lyophilyzed powder used as the starting material in the isolation of erythrocuprein.

rocyte copper (9); 4-ml aliquots of the erythrocyte suspension were hemolyzed by freeze-thawing three times and diluted to 25 ml with saline. The diluted hemolysate was cleared of stroma by centrifugation at 15,000 G for 30 minutes and stored at −40° C.

The erythrocuprein content of a 1-ml aliquot of the hemolysate was determined by the quantitative precipitin technic (10). The anti-erythrocuprein serum (AS-II) was calibrated with erythrocuprein (E-II), as described previously (1). Erythrocuprein was calculated from the calibration curve by the use of an erythrocuprein nitrogen value of 16.53 per cent. The copper in the erythrocyte associated with erythrocuprein was calculated by assuming an erythrocuprein copper content of 0.34 per cent (2).

Evaluation of the immunochemical estimation of erythrocuprein. When erythrocuprein was added to hemolysates and the erythrocuprein concentration of each hemolysate was determined before and after addition of purified erythrocuprein, between 88 and 95 per cent of the added erythrocuprein was recovered. In an experiment designed to determine whether addition of copper to an erythrocyte suspension *in vitro* might influence the determination of erythrocuprein, no change was found in the erythrocuprein level, although the concentration of copper in the erythrocytes was increased 650 per cent.

To estimate the reproducibility of the immunochemical method for the measurement of erythrocuprein, blood was drawn from 20 normal male and female subjects, and the values obtained for duplicate samples were compared. The mean difference between duplicates was 2.7 per cent, with a range of 0 to 8.6 per cent. Aliquots were taken periodically over a 4-month period from a sample of diluted hemolysate during storage at −40° C. A definite trend of change in values with the period of storage was not observed.

Radioisotope incorporation studies. Copper^{64} was obtained from the Oak Ridge National Laboratories as cupric nitrate in 1 M nitric acid. Sodium hydroxide (5 N) was added to precipitate cupric hydroxide. The precipitate was washed with distilled water to remove sodium nitrate. The moist precipitate was dissolved in acetic acid and the solution evaporated to dryness. The dry cupric^{64}-acetate (specific activity of approximately 600 mc per g) was dissolved and diluted with 0.85 per cent sodium chloride to a concentration of approximately 3 μc per ml.

All radioactive samples were counted in a well-type, thallium-activated sodium iodide crystal scintillation counter with an efficiency of 5 per cent for copper^{64}. The radioactivity was expressed in counts per minute, corrected for background and decay. All batches of radiocopper were checked for decay rate, which was found to correspond to the expected half-life of 12.8 hours for copper^{64}.

Fifty ml of freshly drawn blood was added to 12 ml of acid citrate dextrose anticoagulant solution; 1 ml of cupric^{64}-acetate solution was then added. The mixture was incubated under sterile conditions at 37° C with constant shaking for various periods of time.

For the study of the transfer of radiocopper between plasma, erythrocytes and erythrocuprein, 2-ml aliquots of the incubation mixture were pipetted in duplicate into counting vials. Each vial was centrifuged and the plasma removed with care to avoid loss of erythrocytes. The cells were washed twice with saline, with the same precautions, and the packed cells were then frozen and thawed three times. The hemolysate was diluted to the original volume with saline and its radioactivity was determined. Throughout these steps the erythrocytes remained in the original counting vial.

For the study of the transfer of radiocopper between plasma and erythrocuprein, the procedure for the quantitative immunochemical measurement of erythrocuprein was modified to allow rapid precipitation of the antigen because of the short half-life of copper^{64}. After the radioactivity of the hemolysate had been determined, the hemolysate was transferred to a centrifuge tube. The erythrocuprein was precipitated by incubation of the hemolysate with 2 ml of antiserum at 37° C for 2 hours. After cooling to 4° C, the precipitate was collected and washed twice with cold saline. The precipitate was then dissolved in 1 N NaOH, the solution was diluted to 2 ml after transfer to a counting vial, and the radioactivity was determined. Supernatant tests indicated that only about 70 per cent of the erythrocuprein was precipitated under the conditions of these experiments.

To determine the specificity of the immunochemical precipitation of labeled erythrocuprein, the ability of normal rabbit serum and a foreign immune system to precipitate copper^{64} from a labeled hemolysate was tested. When 2 ml of normal rabbit serum was added to the hemolysate, no precipitate formed. Addition of 2 ml of rabbit anti-egg albumin serum, followed by an amount of crystalline egg albumin calculated to bring the system to the equivalence point, led to the formation of a copious precipitate. After washing with cold 0.85 per cent sodium chloride, the precipitate contained less than 3 per cent of the hemolysate radioactivity.

The method of chromatography used for the preparation of erythrocuprein was modified for the partial separation of copper^{64}-containing substances from a labeled stroma-free hemolysate. Diethylaminoethyl cellulose (DEAE-SF, California Biochemical Foundation) was washed with 1 N NaOH followed by 1 M NaCl. It was then washed with 0.2 M sodium phosphate buffer, pH 6.0, until the eluate became free of chloride ion, then with demineralized water until free of phosphate ion and finally with 0.01 M sodium phosphate, pH 6.0. Twelve ml of hemolyzed packed cells, prepared from blood exposed to copper^{64}-acetate for 8 hours at 37° C, was centrifuged at 144,000 G for 1 hour at 5° C to remove stroma. Five ml of the clear supernate was dialyzed, with stirring at 5° C for 1 hour, against 500 ml of 0.01 M sodium phosphate, pH 6.0. The dialysis was repeated twice and the equilibrated hemolysate was chromatographed on a 25 × 190 mm column of DEAE-celulose. Because of the short half-life of copper^{64}, a metering pump (Milton-Roy Minipump) was used to achieve an optimum flow rate of 60 ml per hour.

TABLE I

Volume of packed red cells, serum copper, total erythrocyte copper and erythrocuprein in normal subjects

Group	Vol. packed red cells	Serum copper	Total erythro. copper	Erythrocuprein	$\frac{\text{E Cu}}{\text{T Cu}} \times 100$*
	ml/100 ml blood	*μg/100 ml serum*	*μg/100 ml erythro.*	*mg/100 ml erythro.*	%
Male					
Mean ± SD	49 ± 2.4	107 ± 11.9	89 ± 8.5	16 ± 4.4	60 ± 20.2
Range	45 – 52	82 – 125	63 – 96	13 – 20	47 – 78
Female					
Mean ± SD	44 ± 2.5	127 ± 15.5	89 ± 12.7	17 ± 7.0	65 ± 24.0
Range	41 – 49	103 – 158	72 – 107	13 – 26	48 – 82
Both groups					
Mean ± SD	47 ± 3.4	117 ± 17.1	89 ± 11.4	16 ± 2.9	62 ± 11.0
Range	41 – 52	82 – 158	63 – 107	13 – 26	47 – 82

* E Cu, erythrocuprein copper (erythrocuprein in mg × 3.4); T Cu, total erythrocyte copper.

Ten-ml fractions of the effluent were collected and the absorbance at 280 mμ and the copper[64] content were determined. The column was washed with 0.01 M phosphate buffer, pH 6.0, until ultraviolet-absorbing materials were no longer eluted. A phosphate concentration gradient to 0.2 M sodium phosphate, pH 6.0, was then used to elute other materials from the column.

Carboxymethyl-cellulose (CM-W, California Biochemical Foundation) was washed (5) and equilibrated with 0.01 M sodium phosphate buffer, pH 6.0. All fractions containing copper[64] that were eluted with 0.01 M phosphate from DEAE-cellulose were pooled and applied to a 25 × 600 mm column of CM-cellulose. The CM-cellulose was eluted in successive steps with 0.01 M sodium phosphate buffers at pH 6.2, 7.0, 7.3, 7.5, and 8.4. Ten-ml fractions of the effluent were collected and analyzed as described above.

Other methods and material. Hematologic studies were performed by conventional methods (11). Serum copper was measured by the method of Gubler and co-workers (12) and carbonic anhydrase by the method of Keilin and Mann (13).

The normal subjects used in these investigations were laboratory and office personnel between the ages of 20 and 45. Blood samples were drawn from the individuals without regard to time of day or recent dietary intake.

RESULTS

Total copper and erythrocuprein in normal human erythrocytes. The volume of packed red cells (VPRC), serum copper concentration, total erythrocyte copper concentration and erythrocuprein concentration were determined in a single sample of blood from each of 20 normal subjects. The results are presented in Table I. The values for VPRC, serum copper and total erythrocyte copper are within the normal range of values reported previously from this laboratory (4, 9, 11).

The mean erythrocuprein concentration in normal individuals was 16 mg per 100 ml of packed erythrocytes. When the copper bound to erythrocuprein was calculated from the erythrocuprein concentration, slightly more than half of the erythrocyte copper was present as erythrocuprein copper. No significant difference between the sexes was noted in this regard.

To determine the degree of variation in total erythrocyte copper and erythrocuprein from week to week in a single individual, these two parameters were measured in two male and two female subjects (Figure 3). The variation in both measurements in a single individual during a period of 1 month approximated the variation found in the entire group of normal subjects and was greater than that found in replicate samples. A correlation was not observed between erythrocuprein and total erythrocyte copper in 41 paired determinations ($R = +0.23$; $p > 0.1$).

Total erythrocyte copper and erythrocuprein in erythrocytes from patients with alterations in the concentration of serum copper. To investigate the influence of the level of serum copper on the concentrations of total erythrocyte copper and erythrocuprein, serum copper, total erythrocyte copper and erythrocuprein were measured in 17 patients with conditions known to be associated with alterations in serum copper (Table II). In general, in spite of wide variations in serum copper (10 to 322 μg per 100 ml), the total erythrocyte copper and erythrocuprein concentrations did not differ from the normal. In one of the two patients with Wilson's disease and in one of the patients

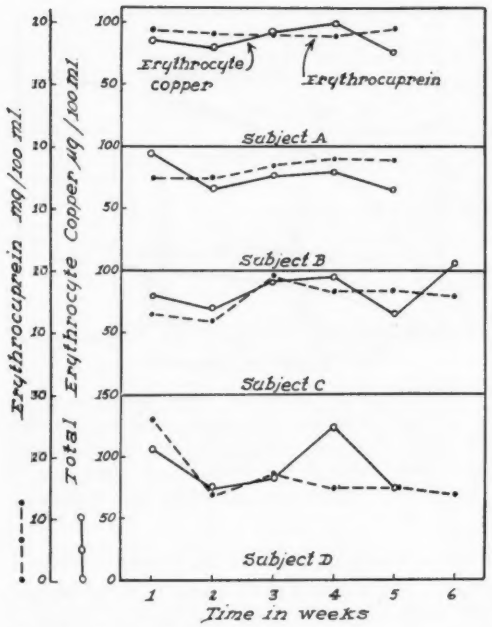

FIG. 3. THE VARIATION IN TOTAL ERYTHROCYTE COPPER AND ERYTHROCUPREIN LEVELS MEASURED AT WEEKLY INTERVALS IN FOUR NORMAL SUBJECTS.

with idiopathic hypocupremia (14), the concentration of erythrocuprein was in the low normal range.

The erythrocuprein concentration was measured in 11 patients (3 with thyrotoxicosis, 2 with iron deficiency anemia, 1 with aplastic anemia, 1 with lymphosarcoma, 1 with paroxysmal nocturnal hemoglobinuria, 1 with pyridoxine-responsive anemia, 1 with biliary cirrhosis, and 1 with malignant melanoma) in addition to those presented in Table II. The concentration of erythrocuprein was within the normal range in each case.

Immunochemical identity of erythrocuprein. To determine whether qualitative immunologic differences in erythrocuprein could be detected among individuals, hemolysates were tested with rabbit anti-erythrocuprein serum by the agar double diffusion technic. Hemolysates from 7 normal subjects, 105 patients from the hematology clinic, 40 patients from the wards of the medical and pediatric services and from 3 samples of umbilical vein blood were studied in this fashion. In every case a single zone of precipitation was observed which showed a reaction of identity with purified erythrocuprein. No instance of cross reaction or absence of the zone of precipitation was observed.

Because cuproproteins have been isolated from species other than man (15), erythrocytes from pigs, rabbits, chickens and steers were tested for the presence of erythrocuprein by the agar double diffusion technic. No reaction was found between antihuman erythrocuprein serum and hemolysates from these species. When we used rabbit antihuman erythrocuprein serum to compare a hemolysate of erythrocytes, obtained from a squirrel

TABLE II

Total erythrocyte copper and erythrocuprein in conditions associated with alterations in the concentration of serum copper

Condition	Patients	Vol. packed red cells	Serum copper	Total erythro. copper	Erythro-cuprein
	no.	*ml/100 ml*	*µg/100 ml*	*µg/100 ml*	*mg/100 ml*
Normal subjects	20	47 ± 3.4 (41 – 52)*	117 ± 17.1 (82 – 158)	89 ± 11.4 (63 – 107)	16 ± 2.9 (13 – 26)
Pregnancy	10	38 ± 3.6 (34 – 43)	246 ± 41.0 (187 – 322)	75 ± 13.6 (54 – 100)	19 ± 4.4 (15 – 26)
Umbilical vein blood	3	59 49	68 72 57	104 109 95	26 15 15
Wilson's disease	2	54 46	28 18	89 67	12 21
Idiopathic hypocupremia of infancy	2	18 26	10 52	162 78	13 12

* Range.

monkey (*Saimiri sciureus*), with purified erythrocuprein from human subjects, a reaction of partial identity was observed.

Because cuproproteins have been demonstrated in mammalian tissues (16, 17), homogenates of human brain, liver and kidney were tested with rabbit anti-erythrocuprein serum. A single precipitin band identical with erythrocuprein was observed in each tissue.

Because the sensitivity of the agar diffusion tests was sufficient to show precipitin bands due to small amounts of blood trapped in the organ before homogenization, quantitative precipitin tests were performed on the supernates obtained from the homogenized tissues (Table III). When the erythrocuprein concentration was corrected for trapped erythrocyte erythrocuprein by calculation from the concentration of hemoglobin in the homogenate, only 1 per cent or less of the total erythrocuprein could be accounted for by the trapped erythrocytes. The erythrocuprein copper in no case represented more than 10 per cent of the total copper found in the sample.

Transfer of copper^{64} between plasma, erythrocytes and erythrocuprein. To determine whether copper added to plasma could be detected in erythrocuprein, copper^{64}-acetate was incubated with whole blood at 37° C for 12 hours. Aliquots of the incubation mixture were taken at 2, 4, 8 and 12 hours for determination of radioactivity in the mixture, in erythrocytes and in erythrocuprein (Figure 4).

In confirmation of earlier studies (18), there was a rapid transfer of copper from plasma to erythrocytes; 82 per cent of the radioactivity added to whole blood appeared in the erythrocytes in 12 hours. There was a slow transfer of radiocopper into erythrocuprein during this period. About 25 per cent of the radioactivity added was in erythrocuprein after 12 hours, but as mentioned previously, under the conditions of this experiment not all of the erythrocuprein was precipitated by the antiserum.

TABLE III

Erythrocuprein in liver, brain and kidney

Organ	Diagnosis	Erythrocuprein	
		μg/g*	mg/organ
Liver	Myocardial infarction	150	233
	Cerebrovascular accident	180	171
	Congestive heart failure and uremia	260	273
	Pyelonephritis	1,240	1,116
	Wilson's disease	67	34
	Wilson's disease	170	244
Brain	Myocardial infarction	50	67
	Huntington's chorea	80	112
Kidney	Myocardial infarction	90	32

* Wet tissue.

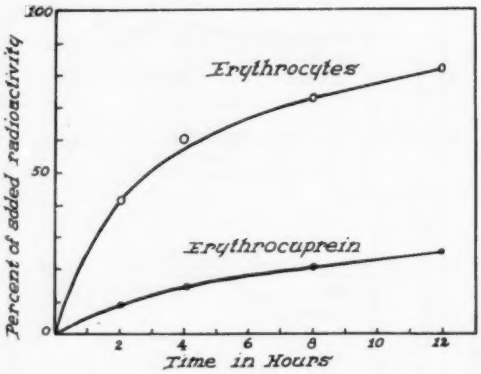

FIG. 4. TRANSFER OF COPPER^{64} FROM PLASMA TO ERYTHROCYTES AND ERYTHROCUPREIN, AFTER *in vitro* INCUBATION. The uptake curves are expressed as per cent of the total radioactivity added to the whole blood which was recovered in the erythrocytes and in the erythrocuprein.

Separation of a second soluble erythrocyte copper component from erythrocuprein. Since the studies on the quantitative measurement of erythrocuprein indicated that all of the copper in erythrocytes could not be accounted for in this cuproprotein, an effort was made to separate a second soluble copper component from erythrocuprein. Copper^{64}-acetate was incubated with whole blood for 8 hours at 37° C. The erythrocytes were separated from the plasma, washed, and hemolyzed. The stroma was separated from the soluble hemolysate by centrifugation at 144,000 G for 1 hour at 5° C. An aliquot of the labeled stroma-free hemolysate was subjected to chromatography on DEAE-cellulose (Figure 5). The fractions under the initial peak, eluted from the column by 0.01 M phosphate buffer, were pooled and were found to contain hemoglobin, carbonic anhydrase activity, and 50 per cent of the radiocopper of the hemolysate.

At approximately the midpoint of the gradient, a peak of radioactivity appeared which contained about 42 per cent of the radioactivity of the

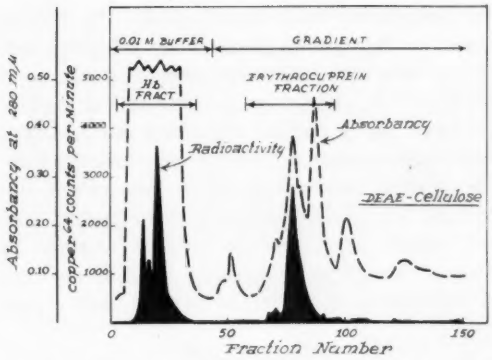

FIG. 5. CHROMATOGRAM OF A HEMOLYSATE OF LABELED HUMAN ERYTHROCYTES. Erythrocytes were incubated with copper[64] for 8 hours, washed, packed, and hemolyzed. Stroma was removed by centrifugation; 10 ml of the clear hemolysate was dialyzed against 0.01 M phosphate buffer (pH 6.0) and chromatographed on DEAE-cellulose. The values on the ordinate refer to the corrected cpm found in each 10-ml effluent fraction.

stroma-free hemolysate. The fractions representing the peak were pooled and an aliquot was incubated with 2 ml of anti-erythrocuprein serum for 2 hours at 37° C. All of the radioactivity in the aliquot was precipitated by the specific antiserum. In three experiments, from 83 to 100 per cent of the total radioactivity added to the column was recovered in the fractions collected under the two peaks.

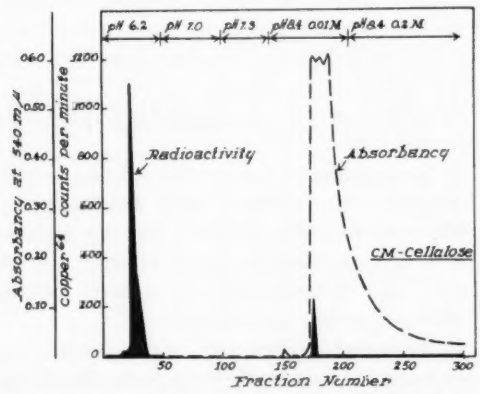

FIG. 6. CHROMATOGRAM OF THE NON-ERYTHROCUPREIN FRACTION OF LABELED HUMAN ERYTHROCYTES. The fractions from the DEAE-cellulose column (Figure 5) containing the initial peak of radioactivity were chromatographed on CM-cellulose without further treatment. The values on the ordinate refer to the corrected cpm found in each 10-ml effluent fraction.

When the pooled fractions representing the initial peak of radioactivity eluted from the DEAE-cellulose were subjected to chromatography on CM-cellulose, all the copper[64] recovered from the CM-cellulose appeared immediately in the pH 6.2 effluent (Figure 6). This effluent contained all the carbonic anhydrase activity of the original hemolysate. Most of the hemoglobin was adsorbed by the CM-cellulose under these conditions, although the effluent exhibited slight absorbance at 540 mμ. The fractions representing the radioactive peak were pooled, dialyzed, and lyophilized. The combined fractions contained no erythrocuprein as determined immunochemically. When this material was tested against antiserum to the crude starting material (LP) by the agar double diffusion technic, three antigens were demonstrated. Four protein components were demonstrated by starch gel electrophoresis at pH 9.5. Further purification of the radioactive component(s) of this fraction was not feasible because of the short half-life of copper[64].

DISCUSSION

The results of this study indicate that normal human erythrocytes contain 16 ± 2.9 mg of erythrocuprein per 100 ml of packed cells. Assumed that erythrocuprein contains 3.4 μg of copper per mg of protein (2), erythrocuprein accounts for about 60 per cent of the copper present in erythrocytes. These values are lower than those reported previously (1). In the previous study the erythrocuprein was purified by tricalcium phosphate gel adsorption, and ethanol and ammonium sulfate fractionation. It has now been shown that this material contained a small amount of highly antigenic impurity. The erythrocuprein used in the present study was purified by chromatography and a contaminating antibody could not be demonstrated in the antiserum prepared by the use of this more highly refined preparation.

In agreement with the observation that erythrocuprein does not account for all of the copper in erythrocytes, a second soluble copper component(s) has been separated from hemolysates by column chromatography. The nature of this second copper-containing component(s) is unknown.

The existence of two erythrocyte copper pools was first suggested by studies in copper-deficient

swine (19). It was noted that the concentration of copper in the erythrocytes of such swine decreased rapidly early in the course of the deficiency from a level of 110 μg per 100 ml of packed cells to approximately 67 μg. Thereafter, in spite of the severity of the copper deficiency there was little further decrease in the erythrocyte copper concentration. Bush and colleagues (18) noted in a later study that when radiocopper was incubated with normal human erythrocytes for 15 to 30 minutes, radiocopper moved rapidly into and out of the erythrocytes; this fraction of erythrocyte copper was referred to as the "labile pool." However, when erythrocytes were incubated with radiocopper for 24 hours, the radiocopper was transformed in part into a fraction that turned over much more slowly; the copper in this fraction was referred to as the "stable pool."

In the same study (18) it was also demonstrated that, when radiocopper was added to plasma, it entered the "direct-reacting" fraction of plasma copper and did not exchange with the copper in ceruloplasmin. From the studies on the incubation of erythrocytes with radiocopper reported in this paper, it is apparent that radiocopper added to plasma does move into erythrocuprein and into the second fraction.

The studies on the erythrocuprein concentration in erythrocytes from patients with various disease states indicate that the erythrocuprein content is quite constant. In patients with hypercupremia, the concentration of erythrocuprein was not increased. At most only an equivocal reduction in erythrocuprein was observed in patients with severe hypocupremia. Thus, the amount of erythrocuprein in erythrocytes was independent of the ceruloplasmin concentration, since about 95 per cent of the copper in serum is in ceruloplasmin. The failure to detect qualitative differences between individual erythrocupreins from over 150 subjects with a wide variety of blood dyscrasias when tested by agar double diffusion suggests that qualitative as well as quantitative alterations in erythrocuprein in disease are not common, if such do occur.

Erythrocuprein was detected in human liver, kidney and brain in concentrations greater than could be accounted for by the number of erythrocytes present, but the copper in the erythrocuprein fraction of the organs accounted for less than 10 per cent of the total organ copper. Erythrocuprein, on the basis of physicochemical properties, is quite similar to the brain copper protein, cerebrocuprein I, isolated by Porter and Folch (17), but until highly purified cerebrocuprein has been tested against anti-erythrocuprein serum, the identity of these two cuproporteins must remain uncertain. Erythrocuprein can be distinguished from the human hepatocuprein, isolated recently by Shapiro, Morell and Scheinberg (20), on the basis of differences in molecular weight and copper content.

SUMMARY

1. A chromatographic method for the preparation of immunologically homogeneous erythrocuprein has been described. The physical and chemical properties of the erythrocuprein isolated were similar to those of the erythrocuprein isolated previously.

2. The erythrocuprein concentration was determined by the precipitin technic in erythrocytes from 20 normal subjects. The mean value ± 1 SD was 16 ± 2.9 mg per 100 ml of packed cells. Since erythrocuprein contains 3.4 μg of copper per mg of protein, the copper in erythrocuprein accounts for about 60 per cent of the total erythrocyte copper.

3. Quantitative and qualitative study of erythrocuprein in erythrocytes from patients with a variety of disorders failed to disclose alterations from the normal. The concentration of erythrocuprein was independent of alterations in the concentration of serum copper.

4. Erythrocuprein was detected in human brain, liver and kidney.

5. Hemolysates of pig, rabbit, chicken and bovine erythrocytes failed to react with antihuman erythrocuprein rabbit serum in the agar diffusion system. A reaction of partial identity was obtained with a hemolysate of squirrel monkey (*Saimiri sciureus*) erythrocytes.

6. A second erythrocyte copper fraction has been separated from erythrocuprein by chromatography on diethylaminoethyl-cellulose and from hemoglobin by chromatography on carboxymethyl-cellulose. The nature of the copper compound(s) in this fraction is unknown.

7. When radiocopper was incubated with whole blood, radioactivity was detected both in the

erythrocuprein and in the second copper-containing fraction.

ACKNOWLEDGMENTS

We are indebted to Dr. A. Haut for the starch gel electrophoresis studies; to Mr. D. M. Brown for the determination of the molecular weight of erythrocuprein; and to Dr. J. H. Hink, Jr., Cutter Laboratories, Inc., Berkeley, Calif., for the preparation of the crude erythrocuprein starting material (Fraction LP).

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SOME OBSERVATIONS ON THE METABOLISM OF D-GALACTOSE IN NORMAL MAN

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For many years clinical interest in D-galactose centered about the usefulness of galactose tolerance tests in the diagnosis of impaired liver function. Several investigators, employing an intravenous galactose tolerance test, have reported their results (1–3). The monograph by Stenstam (4) is perhaps the most extensive analysis of both the oral and intravenous tolerance test yet reported. In all of these studies the parameter for measurement of galactose metabolism has been the disappearance rate of the sugar from blood or the urinary excretion of the sugar for an arbitrary time interval.

More recently research in galactose metabolism has had as its focal point the disease, congenital galactosemia. Kalckar and Maxwell, and their associates (5–7) have contributed to the delineation of the pathways of galactose metabolism and have pinpointed the enzymatic defect in galactosemia (8, 9). Other investigators have reported that certain steroids as well as menthol are able to prevent the depression of galactose oxidation in liver slices and homogenates caused by the metabolism of ethanol and aldehydes (10–12). The administration of progesterone (13) and menthol (12) has enhanced the ability of galactosemic subjects to oxidize galactose to CO_2.

Because of the latter experiments it became important to delineate the physiologic disposition and metabolic fate of C^{14}-galactose in normal subjects. This report presents the results of experiments performed for this purpose. Observations have been made of the effect of progesterone, menthol, and ethanol on galactose-1-C^{14} oxidation.

METHODS

Sixteen intravenous infusions of galactose-1-C^{14} were carried out in 6 normal male volunteer subjects whose ages ranged from 18 to 22. These subjects were maintained on a 300 g carbohydrate diet and had normal glucose tolerance as determined by the method of Amatuzio, Stutzman, Vanderbilt and Nesbitt (14). All experiments were performed after an overnight fast which was continued during the 5-hour period of study.

All of the above subjects received 5 μc of galactose-1-C^{14} either as a tracer of 1.05 mg or mixed with either 10 or 20 g of unlabeled galactose present in 20 per cent solution. In two studies 50 mg per day i.m. progesterone and in another experiment 750 mg per day p.o. menthol were administered for 5 and 3 days, respectively, prior to the isotope infusion. When ethanol was given, 10 or 20 ml of absolute ethanol was diluted with an equal volume of water and ingested, p.o., 5 minutes prior to the radioactive galactose infusion. At various time intervals after the infusion, expired air for $C^{14}O_2$ analysis was collected in Douglas bags and blood was drawn for galactose and glucose analysis. Urine was collected for 24 hours for determination of radioactivity, and in some experiments in which large quantities of galactose were given, assays were performed for reducing substance and glucose oxidase-reacting material.

Galactose-1-C^{14} (specific activity 4.72 μc per mg) was purchased from Dr. H. Isbell of the National Bureau of Standards and prepared for intravenous use as a solution of 1 μc per ml of normal saline by the radiopharmacy of the National Institutes of Health. Unlabeled galactose was purchased from the Pfanstiehl Co. and prepared for i.v. use as a 20 per cent solution by the NIH pharmacy. These solutions were sterile and pyrogen-free. USP absolute ethanol was used. Progesterone in oil was the commercial preparation of Eli Lilly and Co. L-menthol was given orally as a 25 per cent peanut oil solution in gelatin capsules containing 250 mg of the drug. The Na pyruvate used was purchased from Schwartz Biochemical Co. and prepared for use as a 10 per cent solution in normal saline.

Carbon dioxide was assayed by the method of Fredrickson and Ono (15) and the $C^{14}O_2$ in Hyamine assayed in a Packard Tri-Carb liquid scintillation spectrometer counting at 54 per cent efficiency. Blood glucose in filtrates prepared by the Somogyi procedure and urine glucose were assayed with glucose oxidase.[1] Galactose was estimated as the difference between total reducing substance by the Nelson method (16) and glucose oxidase-positive material. Blood was assayed for radioactivity as described previously (17). Glucose was isolated from blood by the method of Blair and Segal (18). Radioactive galactose in blood was estimated by the isotope dilution technic as follows. To a 1 : 10 Somogyi blood

[1] Glucostat reagent purchased from Worthington Biochemical Co., Freehold, N. J.

filtrate made from 20 ml whole blood was added a known amount (approximately 50 mg) of galactose. The filtrate was evaporated to 3 ml. To this was added an equal volume of concentrated nitric acid. This was heated for 90 minutes in a boiling water bath and then placed overnight in a refrigerator at 4° C. The mucic acid crystallized out of solution and was recrystallized from hot water. Both gluconate and mucic acid were combusted to CO_2 by the method of Van Slyke, Plazin and Weisiger (19). The $BaCO_3$ thus obtained was treated with acid to liberate CO_2 which was trapped in Hyamine and counted by the scintillation technic (20). Urine-C^{14} was assayed as previously described (21).

Two laboratory personnel, one male and one female, ages 33 and 35, respectively, donated blood from which leukocytes were prepared by the method of Skoog and Beck (22). The incubation procedure was similar to that already reported (23). Human liver for *in vitro* study was obtained at surgery from a 29-year old male with metastatic islet cell carcinoma. Tumor was present in the liver but none was observed macroscopically in the segments used to prepare the tissue slices for the *in vitro* study.

RESULTS

The effect of increasing the quantity of sugar injected on oxidation of galactose-1-C^{14} to $C^{14}O_2$. The radioactive sugar was metabolized to labeled CO_2 as shown for a tracer amount in Figure 1. The control curve in the upper part of Figure 1 is similar to that observed after glucose-1-C^{14} administration (24). Table I reveals the per cent of the administered C^{14} found in expired air at hourly intervals after injection of the isotope. When the amount of injected galactose was about 1 mg, 31 to 35 per cent of the C^{14} dose was in ex-

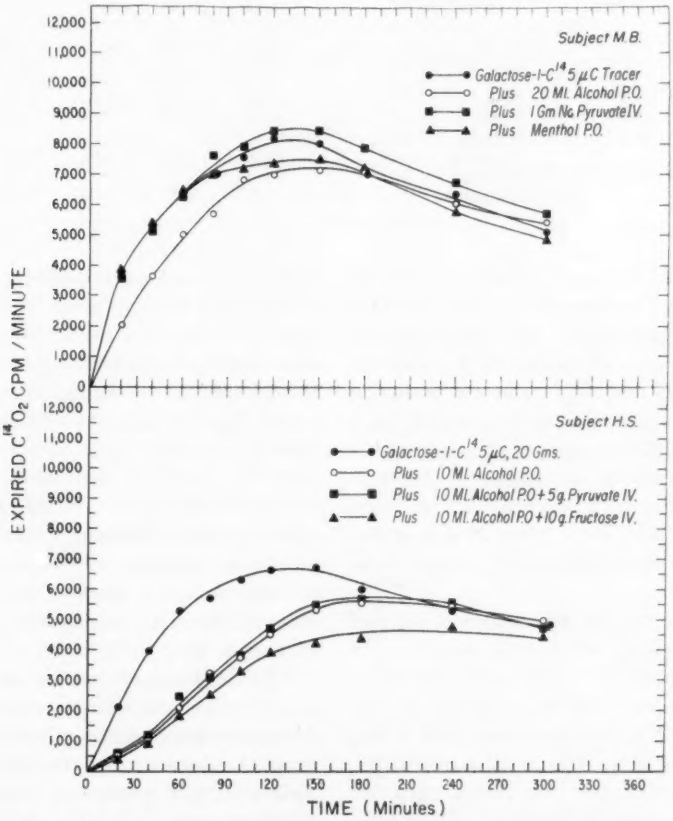

FIG. 1. UPPER PORTION IS THE EXPIRED $C^{14}O_2$ AFTER I.V. ADMINISTRATION OF A TRACER QUANTITY OF GALACTOSE-1-C^{14} IN A NORMAL SUBJECT. Alcohol refers to ethanol. Lower portion is the $C^{14}O_2$ excretion curves after administration of 20 g galactose containing the same amount of galactose-1-C^{14} as given in the tracer experiments.

TABLE I

The extent of oxidation of galactose-1-C^{14} after intravenous injection into normal subjects

Subj.	Amt. of sugar	Exptal. additions	CO_2 excret.	Administered C^{14} in expired air, Minutes after injection: 60	120	180	240	300
	g		*mmoles/min*			%		
S.K.	0.00106	None	10.8	3.86	10.17	17.60	25.08	31.71
	0.00106	Progesterone	10.8	3.84	10.53	18.23	25.75	32.42
J.F.	0.00106	None	12.2	4.31	12.54	21.25	28.77	35.23
	0.00106	Progesterone	12.7	5.49	14.75	24.41	32.85	39.85
M.B.	0.00106	None	9.9	4.24	11.87	20.09	27.19	33.07
	0.00106	Menthol	10.0	4.24	11.53	19.14	25.80	31.30
	0.00106	Pyruvate, *1 g*	10.6	4.24	12.13	20.69	28.22	34.58
	0.00106	Ethanol, *20 ml*	9.6	2.80	9.19	16.57	23.50	29.43
D.S.	10.0	None	10.3	2.01	7.20	14.40	22.09	29.11
	10.0	Ethanol, *10 ml*	10.5	1.02	5.23	11.62	18.45	24.93
	Glucose-1-C^{14}							
	0.0018	None	10.6	4.02	12.50	21.61	29.31	35.56
	0.0018	Ethanol, *10 ml*	11.1	4.69	14.01	23.42	31.58	38.54
D.P.	20.0	None	11.7	1.85	6.66	12.75	19.10	25.24
	20.0	Ethanol, *20 ml*	13.0	0.77	3.19	7.93	14.03	20.11
H.S.	20.0	None	10.1	3.08	9.49	16.37	22.51	27.78
	20.0	Ethanol, *10 ml*	10.7	0.94	4.47	9.98	15.81	21.27
	20.0	Ethanol, *10 ml* Fructose, *10 g*	9.7	0.81	3.84	8.30	13.14	17.84
	20.0	Ethanol, *10 ml* Pyruvate, *5 g*	10.5	1.02	4.73	10.44	16.41	21.83

pired CO_2 within 5 hours. This corresponds quite closely with the amount of $C^{14}O_2$ excreted after glucose-1-C^{14} injection as reported previously (24) and shown also in Subject D.S., Table I. The injection of 10 and 20 g sugar containing an identical amount of radioactivity resulted in a slight reduction of the per cent of the injected C^{14} excreted in expired air, as shown in Table I. Subject D.S., given 10 g, excreted 29 per cent, and Subjects D.P. and H.S., given 20 g, excreted 25 and 28 per cent of the radioactivity, respectively, in 5 hours.

The effect of increasing the amount of injected sugar is most striking when one compares the per cent C^{14} excreted at 1 hour with the values for the tracer studies. The lower portion of Figure 1 shows the $C^{14}O_2$ excretion curve after a 20 g infusion in Subject H.S. The initial rate of $C^{14}O_2$ production is about 40 per cent slower than that seen for the tracer study in Figure 1. With time, however, the $C^{14}O_2$ excretion after a load approaches that seen after a tracer quantity. Considering the fact that the specific activity of the injected galactose is lowered 10- and 20-thousand-fold in the load experiments, it appears that the system has a large capacity for metabolizing galactose. The fact that the $C^{14}O_2$ excretion is somewhat lower after a load suggests that the system is beginning to be saturated, for it would be expected that the per cent of substrate metabolized remains constant with increasing concentration until the point of saturation where the value would then decrease. The effect on expired $C^{14}O_2$ observed with increasing quantity of injected galactose is in marked contrast to that observed with D-ribose, where a marked decrease in oxidation occurred upon increasing the sugar given from a tracer to 20 g (17).

Effect of progesterone and menthol on galactose oxidation. It has been reported that progesterone administration to three galactosemic children and menthol administration in two others enhanced their ability to oxidize a tracer quantity of labeled galactose (12, 13). In these experiments, during control periods, virtually no $C^{14}O_2$ was produced, whereas after the administration of these drugs, from 7 to 17 per cent of the C^{14} injected appeared in CO_2 in 5 hours. Three studies, two

with progesterone and one with menthol, have now been performed in normal subjects, and the results are shown in Table I and Figure 1. A problem presented itself in these studies. Whereas, in the galactosemic children in whom the capacity for metabolizing galactose to CO_2 is essentially nil and the effects of a drug stimulating oxidation may be easily seen, in the normal there is a baseline of rapid and extensive metabolism which is probably maximal. Fluctuations in this baseline could make interpretation of the drug studies difficult. In Subject S.K. the per cent of the C^{14} given that appeared in expired air was virtually identical before and after progesterone administration. Subject J.F. showed a slight increase after the drug. Subject M.B., whose post-menthol excretion curve is seen in Figure 1, had no stimulatory effect of the drug. The results indicate that these drugs do not have an effect on galactose metabolism in a normal subject.

Caution must be exercised, however, in comparing the effect of these drugs in post-pubertal normal subjects and children. Because of the problems involved, we have not performed these experiments with radioisotopes in normal children.

Effect of ethyl alcohol on galactose oxidation. Stenstam (4) has demonstrated very clearly in the human that ethyl alcohol impairs the clearance of administered galactose from blood. Several reports have appeared which demonstrate *in vitro* that ethanol inhibits galactose metabolism and that this effect is due to changes in intracellular reduced diphosphopyridine nucleotide (DPNH) (11, 25, 26).

The present studies reveal that small amounts of ethanol may markedly inhibit the metabolism of galactose-1-C^{14} to $C^{14}O_2$. These results are tabulated in Table I. Figure 1 demonstrates this inhibition in a tracer study in Subject M.B. The potency of the effect is brought out here, since this reveals the inhibition of metabolism of 1 mg in a system that has a capacity for metabolizing many thousands of milligrams. The effect of ethanol was greater in the loading experiments with 10 and 20 g. Figure 1 also shows the marked inhibition of appearance of $C^{14}O_2$ in a study with 20 g of the sugar. This effect of ethanol is not seen when C^{14} glucose is injected, as is shown in Table I, Subject D.S.

Smith and Newman (27) have demonstrated in the rat that injected pyruvate decreases the high levels of DPNH generated in liver during ethanol administration by being reduced to lactate with DPNH as a cofactor. Two experiments were therefore performed on the effect of pyruvate on galactose-1-C^{14} oxidation. In the first (Figure 1), 1 g of pyruvate was given i.v. in the absence of ethanol to see whether the normal metabolism could be stimulated. Little or no effect was observed. In the second, shown in the lower section of Figure 1, an attempt was made to reverse the effect of 10 ml of ethanol by injecting 5 g of sodium pyruvate. This amount of pyruvate failed to overcome the inhibition. Because the principal fate of pyruvate in man may be oxidation rather than the conversion to lactate, which would be needed for alteration of DPNH levels, a pyruvate-generating system, involving fructose metabolism, was employed (28). Figure 1 also demonstrates that 10 g of fructose i.v. was also unable to overcome the ethanol effect.

The disappearance of C^{14} and galactose from blood and the conversion of galactose to glucose. Figure 2 demonstrates the change with time in whole blood C^{14} after a tracer dose of galactose-1-C^{14} was given to Subjects M.B. and J.F. The inset shows the decrease in chemically determined galactose in blood of M.B. after a 5 g infusion. Whereas there was no detectable galactose in blood 15 minutes after the 5 g infusion, at the same time and for many minutes thereafter, much C^{14} was present in the tracer experiment. The rate of decrease of C^{14} was very much slower than that of galactose itself.

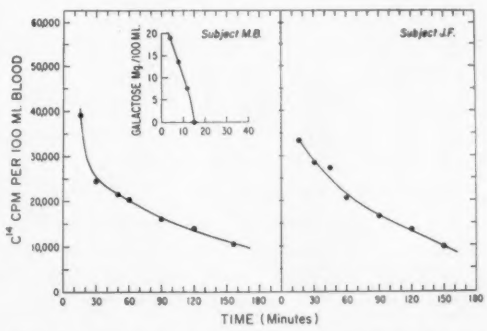

FIG. 2. THE CHANGE IN BLOOD C^{14} WITH TIME AFTER LABELED GALACTOSE INJECTION. The inset shows the disappearance from blood of chemically determined galactose after a 5 g injection.

TABLE II

Distribution of C^{14} in blood after galactose-1-C^{14} and glucose-1-C^{14} administration

Sugar injected and subject	Time after inject.	C^{14} in blood		
		Total C^{14}	Galactose C^{14}	Glucose C^{14}*
	min	*dpm/100 ml*	*dpm/100 ml*	*dpm/100 ml*
Galactose-1-C^{14}				
M.B.	30	48,400	2,723	18,485
	121	21,000	223	7,305
J.F.	15	43,400	3,402	12,904
	96	20,600	657	5,980
Glucose-1-C^{14}				
D.S.	30	35,600		29,680
	90	22,380		10,250

* Corrected for adsorption of radioactive galactose (18).

The latter fact suggested that metabolites of galactose were present in blood. Since the pathway of galactose metabolism involves conversion to glucose, two experiments were carried out to ascertain how much of C^{14} in blood after a tracer injection was in glucose or galactose. The galactose-C^{14} was isolated as mucic acid by a carrier-dilution technic, and glucose as gluconate. Table II contains the results of these experiments. In J.F. 15 minutes, and in M.B. 30 minutes, after the injection, less than 10 per cent of the blood-C^{14} is galactose. At the latter time intervals shown, galactose-C^{14} is even a smaller fraction of the total C^{14}. On the other hand, a substantial amount of the C^{14} is present as glucose. The nature of the other C^{14} compound(s) is unknown. For comparison, results are shown in the table for the amount of C^{14} in blood glucose after glucose-1-C^{14} injection. With time a substantial amount of non-glucose-C^{14} appears in blood, paralleling the results in the galactose experiments.

It is possible to estimate the fraction of injected galactose-C^{14} circulating as glucose by determining the total C^{14} in the glucose compartments. This may be calculated if the specific activity of blood glucose and the size of the glucose pool are known. For the 30-minute point of Subject M.B. the specific activity of blood glucose was 52,700 dpm per mmole. Estimating the glucose pools to be 100 mmoles (24), it may be calculated that about 5 million dpm of the 11 million injected are in body glucose pools. This result is consistent with the known pathway of galactose metabolism in which glucose phosphates are intermediates in the conversion of galactose to CO_2.

In Figure 3 are shown data of the effect of ethyl alcohol on galactose disappearance from blood after a 20 g infusion. As has been demonstrated previously by Stenstam (4), alcohol slows this disappearance and this was not corrected by infusion of 5 g sodium pyruvate, paralleling the results on $C^{14}O_2$ described previously in the section on $C^{14}O_2$ excretion. Figure 3 also shows a rise in blood glucose levels after galactose infusion and this is consistent with the data above on the conversion of galactose to glucose. It should be noted that when alcohol is ingested there is little or no increase in blood glucose, as would be expected if alcohol inhibited the conversion of galactose to glucose.

In certain normal individuals (4, 29) and in galactosemic infants (30), galactose infusion may cause hypoglycemia. Pozza, Galansino, Hoffeld and Foa (31) have demonstrated that galactose infusion causes increased insulin release from the pancreas. Hyperreactivity to insulin may explain the resultant hypoglycemia in these people. The alternative explanation of Sidbury (32) and Ginsburg and Neufeld (33) that phosphoglucomutase is inhibited by accumulated galactose-1-phosphate, thereby slowing glycogen breakdown to glucose, also should be entertained.

Urinary excretion of C^{14}, reducing substance and glucose. Table III presents data on the appearance of C^{14} in urine. About 3 per cent of the injected C^{14} was excreted by this route in the tracer studies. In the one study with menthol (Subject M.B.) significantly less C^{14} appeared in urine. With injection of 20 g, 5 to 8 per cent of the C^{14} originally given was excreted. Alcohol in-

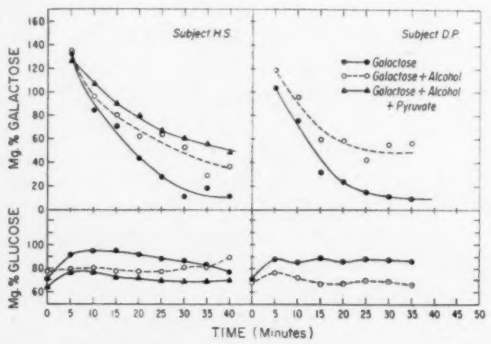

FIG. 3. DISAPPEARANCE OF CHEMICALLY DETERMINED GALACTOSE FROM BLOOD AFTER INFUSION OF 20 G AND THE CONCOMITANT CHANGE IN BLOOD GLUCOSE.

TABLE III

Urinary excretion of C^{14}, reducing substance and glucose oxidase-reacting substance after galactose administration

Subj.	Amt. of galactose inj.	Drug	*Hours:*	Dose excreted: C^{14} 0–5	C^{14} 5–24	Reducing substances 0–5	Reducing substances 5–24	Glucose oxidase reactant 0–5	Glucose oxidase reactant 5–24
	g			%		%		%	
M.B.	0.00106	None		2.7	0.7				
	0.00106	Menthol		0.3	0.6				
	0.00106	Alcohol, *10 ml*		3.2	0.5				
	0.00106	Pyruvate, *1 g*			3.6*				
J.F.	0.00106	None		1.6	1.6				
	0.00106	Progesterone		1.8	0.8				
D.S.	20.0	None		4.5	0.7	7.8	8.3	0.42	0.55
	20.0	Alcohol, *10 ml*		8.6	0.5	13.3	3.1	0.20	0.34
D.P.	20.0	None		7.1	0.9	11.2	3.6	0.31	2.76
	20.0	Alcohol, *20 ml*		17.7	0.7	17.6	1.4	0.53	0.96

* Total 24-hour excretion.

gestion essentially doubled the 5-hour C^{14} excretion. Reducing substance was assayed in urine of the subjects receiving 20 g in order to see whether the C^{14} excreted corresponded to copper-reducing sugar. In all but one urine specimen, reducing substance expressed as galactose was greater than the C^{14}. This led to the suspicion that urinary glucose excretion was elevated and that this might account for the discrepancy between reducing substance and C^{14}. The glucose excretion, however (Table III), was not greater than that reported by Froesch and Renold (34) for normal urine, and did not account for the difference between C^{14} and reducing substance. Froesch and Renold have shown that as much as 1 g of nonspecific reducing substance expressed as glucose was excreted in 24-hour urine specimens. Since the reducing power of galactose is less than glucose, if the reducing substance is expressed as galactose it would represent well over 1 g of the sugar or over 5 per cent of the injected 20 g. Thus, the difference between the C^{14} and galactose expressed as reducing substance could be explained on the basis of nonspecific reducing substance.

In vitro effect of various drugs on galactose oxi-

TABLE IV

*In vitro effect of ethanol, menthol, progesterone, and pyruvate on galactose-1-C^{14} oxidation by human leukocytes and liver**

	cpm/10^6 leukocytes or/100 mg liver†												
		Ethanol 10^{-2} M											
		Menthol				Progesterone				Pyruvate			
	Control	10^{-4}	10^{-5}	10^{-6}	10^{-8} M	10^{-4}	10^{-6}	10^{-8}	10^{-10} M	8×10^{-3}	1.6×10^{-2} M	3.2×10^{-2} M
WBC													
Exp. 1; Subj. A.B.	148	115		112	107		109	100					
Exp. 2; Subj. A.B.	136	103					114	98	113				
Exp. 3; Subj. S.S.	167	126	99			97				250	385	445	
Liver													
Exp. 1; Subj. C.C.	2,736	1,279	2,282	1,340		766							

* Liver slices were prepared with a Stadie-Riggs microtome and placed in modified Warburg flasks containing 2 ml of Krebs-Ringer bicarbonate buffer and 0.24 μc of radioactive galactose. Incubation procedure was similar to that described for the leukocytes (23).

† Leukocyte values are averages of duplicate, and liver the averages of triplicate determinations.

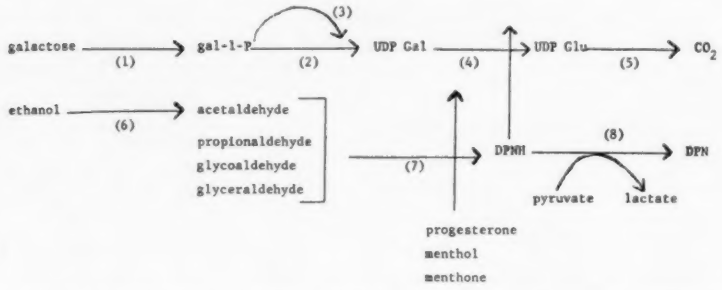

FIG. 4. SCHEMATIC REPRESENTATION OF THE PATHWAY AND FACTORS AFFECTING GALACTOSE METABOLISM. The numbers represent reactions involving the following enzymes: 1, galactokinase; 2, galactose-1-P uridyl transferase; 3, uridinediphosphate galactose (UDPGal) pyrophosphorylase; 4, UDPGal-4-epimerase; 5, uridinediphosphate glucose (UDPGlu) pyrophosphorylase and subsequent enzymes from glucose-1-P to CO_2; 6, alcohol dehydrogenase; 7, aldehyde dehydrogenase; 8, lactic dehydrogenase.

dation by human leukocytes and liver. The inhibition of galactose oxidation in animal liver and intestine by ethanol and certain aldehydes and the reversal of this effect by menthol and progesterone have been reported (10–12). Experiments were performed on human leukocytes to ascertain whether these effects were obtained in human tissues. The data in Table IV demonstrate that ethanol was able to depress galactose oxidation in leukocytes but that menthol and progesterone in a wide range of concentration did not restore the galactose oxidation to normal. On the other hand, pyruvate not only was able to reverse the inhibition caused by ethanol but also caused a stimulation of galactose oxidation above the baseline values. This is in contrast to the lack of effect of pyruvate in the *in vivo* studies described above. Other experiments with normal hemolysates have shown the inability of menthol and progesterone to reverse the depression of galactose metabolism caused by aldehydic substances (35); but when human liver was employed, 10^{-4} M menthol tended to negate the ethanol effect. Progesterone at the same concentration appeared to have no such effect.

DISCUSSION

D-galactose is an important sugar in human nutrition, especially in the young, whose principal food is milk. Milk contains about 5 g per 100 ml of lactose, which is a disaccharide of glucose and galactose (36). It is not surprising, therefore, to find that normal subjects have the capacity to metabolize large quantities of injected galactose.

The pathway of galactose metabolism has been delineated by Caputto and co-workers and Leloir (37, 38) and by Kalckar and Maxwell and associates (5, 6). This is schematically shown in Figure 4. Galactose is phosphorylated to form galactose-1-phosphate (gal-1-P) which is then converted to UDPGal by the enzyme gal-1-P uridyl transferase (reaction 2). This is the deficient enzyme in galactosemia. By an alternate route found in yeast (5), mung bean seedlings (39), and in liver (40) (reaction 3), gal-1-P may be transformed to UDPGal. The physiologic role of this pathway in man remains to be assessed. UDPGal is epimerized by UDPGal-4-epimerase to UDP glucose (reaction 4). The latter may undergo pyrophosphorylytic cleavage to glucose-1-phosphate which then enters the usual pathway of glucose oxidation to CO_2. The present studies show that, in man, galactose is extensively converted to glucose.

Maxwell (41) has described the properties of the UDPGal-4-epimerase enzyme. One of these is marked inhibition by DPNH. DPN is an essential cofactor for the epimerase reaction. It has now become apparent that the level of DPNH in tissue exerts a marked controlling effect on galactose metabolism. Figure 4 summarizes schematically the factors regulating galactose metabolism (10–12, 25, 26). Ethanol via conversion to

acetaldehyde, and various aldehydes via conversion to their respective acids by aldehyde dehydrogenase, generate DPNH (reactions 6 and 7). The DPNH inhibits the epimerization of UDPGal which interferes with galactose conversion to glucose. Progesterone, menthol and menthone in the *in vitro* studies inhibit aldehyde dehydrogenase (reaction 7), thereby preventing the rise in DPNH levels. The effect of ethanol and aldehydes may be reversed by pyruvate conversion to lactate which involves DPNH oxidation to DPN (reaction 8). This takes the "brake" off the epimerase reaction. The importance of the epimerase reaction in the control of galactose metabolism even in the presence of limited amounts of uridyl transferase enzyme (reaction 2) has recently been stressed (42).

The explanation for the lack of an effect in white cells of progesterone and menthol is not readily apparent. Menthol and progesterone are believed to act by affecting the activity of aldehyde dehydrogenase. Maxwell has purified human liver aldehyde dehydrogenase and has found it to be less sensitive to these drugs than are the corresponding enzymes prepared from several species (43). This may be the explanation for an incomplete effect of menthol and the absence of a progesterone effect at the concentrations used in the experiments with human liver. The lack of an effect of the drugs in the white cell could be related to their permeability into the cell. The insensitivity of red cell aldehyde dehydrogenase to these substances (35) tends to suggest, however, that the enzyme present in peripheral blood cells may have characteristics different from the corresponding enzyme present in liver.

Although the role of progesterone and menthol in galactose metabolism in animal tissues *in vitro* has been elucidated, the mechanism of action of these drugs to stimulate galactose metabolism in subjects with galactosemia remains obscure. The explanation of the latter effect becomes more puzzling in view of the present findings that these drugs do not stimulate galactose metabolism in normal subjects. This raises the question of whether it is mere coincidence that progesterone and menthol *in vitro* act primarily in a system depressed by aldehyde oxidation and *in vivo* only in subjects whose metabolism is also depressed.

The knowledge that ethanol ingestion inhibits galactose metabolism has some clinical application. Schwarz has found the accumulation of galactose-1-phosphate in cord blood of a newborn galactosemic baby (44) and, along with others (45), has postulated that there may be *in utero* damage to such a child by transplacental galactose. It might be recommended that when a mother of a known galactosemic child becomes pregnant, in addition to institution of a galactose-free diet, cessation of any ethanol ingestion should be carried out as well.

SUMMARY

Various quantities of galactose-1-C^{14}, when given intravenously to normal human subjects, are rapidly and extensively metabolized to $C^{14}O_2$. This metabolism is markedly slowed by ingestion of ethanol but is not affected by administration of progesterone, menthol or pyruvate. White blood cell metabolism of galactose *in vitro* is inhibited by ethanol, but this inhibition is not reversed by menthol or progesterone. Depression by ethanol of galactose oxidation in human liver *in vitro* is reversed by menthol, as has been observed in other animal species.

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RELATION OF RENIN, ANGIOTENSIN II, AND EXPERIMENTAL RENAL HYPERTENSION TO ALDOSTERONE SECRETION *

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Recent studies (1–3) have provided evidence that a humoral substance secreted by the kidney leads to the hypersecretion of aldosterone which occurs after acute hemorrhage, during chronic Na depletion and during chronic thoracic caval constriction. In subsequent preliminary reports, it has been demonstrated that an aldosterone-stimulating factor is present only in the renin fraction of kidney extracts (4), and that infusions of both renin and angiotensin II result in increased aldosterone output in the dog (5). These findings are consistent with the work of several groups of investigators (6–12), recently summarized by Tobian (13, 14), which suggests that the renin-angiotensin II system plays an important role in the regulation of aldosterone secretion.

The present series of experiments was designed to test the hypothesis that aldosterone secretion is regulated by the renin-angiotensin II system. In the first two experiments the effects of infusions of renin and of synthetic angiotensin II on the pattern of steroid secretion in dogs were studied at several dosage levels; at the smallest dosage level, angiotensin II was infused at a rate too low to affect arterial blood pressure. To determine whether the steroidogenic effects of these agents are dependent upon ACTH release, all experiments were performed in hypophysectomized animals. In the third experiment, aldosterone and corticosterone secretion rates were determined in conscious, unstressed dogs with experimental benign and malignant renal hypertension, and the relationship between the renin content of the hypertensive kidney and aldosterone output was examined.

MATERIALS AND METHODS

In Experiment I, six 16 to 20 kg mongrel dogs were used to study the effects of renin on steroid secretion. Each animal was anesthetized with Na pentobarbital after which hypophysectomy (transbuccal approach), bilateral nephrectomy, and right adrenolumbar vein cannulation were performed. Two hours after hypophysectomy, and approximately 1 hour after nephrectomy, two groups of control samples of adrenal venous blood were collected at 30-minute intervals. All blood collected in these experiments was replaced immediately by homologous transfusion. In one animal, 5 dog units of renin,[1] prepared from dog kidney by the method of Haas and Goldblatt (16), was injected into a foreleg vein; immediately after the injection an infusion of renin was begun at a rate of 0.1 U per kg per minute and continued over the following 40 minutes. In the remaining 5 dogs exactly the same procedure was followed, but a smaller amount of renin (2.2 U as a single initial injection, followed by an infusion of 0.03 U per kg per minute) was given. At least four 10-ml samples of adrenal effluent were collected from 20 to 40 minutes after the onset of the renin infusion. In 5 of the 6 dogs, additional collections of adrenal venous blood were made 30 to 40 minutes after completion of the renin infusion.

In Experiment II the effects of angiotensin II were studied in twenty 15 to 20 kg animals. The dogs were prepared as in Experiment I, except that nephrectomy was performed only in the first 4 and the last 3 dogs of this group. After control collections of adrenal venous blood, angiotensin II in 5 per cent dextrose (valine-5-hypertensin II, Ciba) was infused at different dosage levels. In the first 4 dogs, 20 μg of angiotensin II was injected rapidly into a foreleg vein and was followed by an infusion at a rate of 0.25 μg per kg per minute. In 4 of the simple-hypophysectomized [2] animals, 4.0 μg of angiotensin II was given as an initial injection and was followed by an infusion at a rate of 0.05 μg per kg per minute. The remaining 9 simple-hypophysectomized dogs were given no initial injection of angiotensin II and received only infusions at rates of 0.008 to 0.025 μg per kg per minute. The rate of infusion in each dog was slightly less than that which caused the minimal detectable rise

* The present experimental data were presented, in preliminary form, at the meeting of the Federation of the Societies for Experimental Biology in April, 1961.

[1] As defined by Goldblatt, Katz, Lewis and Richardson, 1 dog U of renin is that amount which, when injected into a trained, unanesthetized, 10 to 20 kg dog, results in a maximal increase in mean arterial pressure of 30 mm Hg within 3 minutes after the time of injection (15).

[2] The "simple-hypophysectomized animal" refers to the dog in which the anterior pituitary has been removed and no other physiological alteration has been made.

in mean arterial pressure. In addition, angiotensin II infusions at rates of 0.008 to 0.013 μg per kg per minute were given to 3 Na-depleted hypophysectomized-nephrectomized dogs; severe Na depletion in these dogs had been achieved by maintenance on a Na-free diet for 2 weeks, during which time each of the animals had received two injections of 2 ml meralluride sodium (Mercuhydrin) intramuscularly. In each of the 20 dogs, at least 4 samples of adrenal effluent were collected 20 to 40 minutes after the beginning of the angiotensin II infusion. In 2 of the animals which received the largest dose and 2 of the animals which were given the intermediate dose of angiotensin II, further collections of adrenal effluent were made 20 to 30 minutes after the completion of the angiotensin II infusion.

In both Experiments I and II, aortic blood pressures were measured by means of indwelling polyethylene catheters and Statham strain gage transducers and recorded continuously on a Sanborn recorder. Aldosterone and corticosterone secretion rates were determined by methods described previously (17), with the double isotope derivative technique of Kliman and Peterson (18). Silber-Porter chromogens were measured by a modification of the technique described by Peterson, Karrer and Guerra (19).

In Experiment III, 15 trained 16 to 20 kg mongrel dogs were used. All animals received (by force feeding when necessary) a synthetic diet containing 60 mEq Na and 18 mEq K per day throughout the period of study. Control blood pressures were measured in the trained, unanesthetized animals by direct femoral arterial puncture with a 20 gage needle connected to a Statham strain gage transducer, and mean arterial pressures were determined and recorded by a Sanborn recording system. After control blood pressure measurements over a 1 to 3 week period, a Goldblatt clamp was placed on the left renal artery of each dog, by the technique of Goldblatt (20). Five to 10 days after left renal arterial constriction a similar clamp was placed on the right renal artery. In the last 6 dogs of the group, in an attempt to produce the experimental malignant hypertensive syndrome, the degree of renal arterial constriction was increased to such an extent that the pulse in the renal artery distal to the Goldblatt clamp was approximately one-fourth of that proximal to the clamp, as determined by digital palpation. In 7 of the 15 animals, serum Na and K determinations were performed by flame photometry both during the control period and 4 to 14 days after application of the second Goldblatt clamp. At intervals varying from 3 days to 3 weeks after the second Goldblatt clamp had been applied, a polyvinyl catheter was placed in the right adrenolumbar vein of each animal under sterile conditions, and three 10-ml samples of adrenal venous blood were obtained.

In 9 of these 15 animals, collections of adrenal effluent were made daily for periods of 3 to 13 days from the conscious, unstressed animal via the chronic indwelling adrenal venous catheter. In one of the animals, the right adrenal venous catheter was removed after 2 weeks of daily collections. In this dog blood pressure measurements were continued over the following 8 weeks, at the end of which time a chronic catheter was placed in the left adrenolumbar vein, by the technique of Hume and Nelson (21); further collections of adrenal effluent were made daily over the following week. Each animal was given 3,000 USP units of heparin immediately before each collection of adrenal venous blood. Since several of the hypertensive animals bled intermittently into the laparotomy site for the first 2 or 3 days after surgery, hematocrits were determined daily throughout the period of study, and each animal received homologous blood transfusions in such quantity as was necessary to maintain the hematocrit at the control level. Because of the well recognized effect of blood loss on steroid production (22, 23), aldosterone and corticosterone secretion rates for each dog were determined only on those days on which the hematocrit approximated the control level.

Four of the animals with benign renal hypertension were anesthetized with Na pentobarbital at intervals of 15, 16, 60, and 90 days, respectively, after the application of the second Goldblatt clamp, and the kidneys were rapidly removed and frozen at $-20°$ C; care was taken to avoid blood loss during removal of the kidneys. The kidneys were also removed from 4 of the dogs with malignant hypertension as soon as possible after the death of the animal; the interval between application of the second Goldblatt clamp and removal of the kidneys varied from 7 to 17 days in these animals. At intervals of 2 to 6 months after freezing, the kidneys were thawed in a water bath at room temperature, and renin was extracted by a modification of the technique of Haas and Goldblatt (16). The modification consisted in acidifying the extract to pH 2.75 (instead of pH 2.60), as this was the pH level at which the maximal yield of renin was obtained from normal dog kidneys in our laboratory. The pH was determined with a model 76 Beckman expanded scale pH meter. Renin extractions of both kidneys of one benign and one malignant hypertensive dog were always performed simultaneously. Assays of the potency of each sample of renin were performed in duplicate or triplicate, with a modification of the technique described by Goldblatt and colleagues (15). The modification consisted in measuring arterial blood pressure by direct femoral arterial puncture with a 20 gage needle attached to a Statham strain gage transducer, and determining mean arterial pressure on a Sanborn recording system.

RESULTS

Effects of renin on steroid secretion in hypophysectomized-nephrectomized dogs

In each animal the control aldosterone, corticosterone and Porter-Silber chromogen secretion rates were in the range previously observed in hypophysectomized-nephrectomized dogs (1, 3) (Table I, see Figure 1). Injection of 5 dog U of renin followed by infusion of renin at a rate of 0.1 U per kg per minute (Figure 1) re-

TABLE 1

Effects of renin on adrenocortical steroid secretion in hypophysectomized-nephrectomized dogs*

Dog	Aldosterone secretion		Corticosterone secretion		Porter-Silber chromogen secretion		Mean arterial blood pressure†		
	Before	During	Before	During	Before	During	Before	2 min after initial injection	During infusion
	μg/min		μg/min		μg/min		mm Hg		
1	0.001	0.006	0.01	0.05	0.15	0.05	60	136	146
2	.005	.017	.16	.18	.15	.23	35	82	82
3	.011	.037	.09	.73	.16	.52	50	95	92
4	.016	.027	.02	.18	.12	.15	60	90	98
5	.004	.008	.01	.09	.03	.06	80	130	130
Average	.007	.019	.06	.25	.12	.20	57	107	110

* In this series of dogs an initial intravenous injection of 2.2 dog units of renin was followed by an infusion of renin at a rate of 0.03 U/kg/minute.

† Values for mean arterial pressure during infusion represent average pressures during the 40-minute period of renin infusion. Note that the blood pressure elevation was sustained throughout the period of renin infusion in every animal.

sulted in a striking increase in secretion rates of each of the steroids studied; the relative increases in aldosterone (4-fold) and corticosterone output (5-fold) were greater than the increment in Porter-Silber chromogen production (2-fold). Despite an increase in mean arterial pressure from 70 to 130 mm Hg, adrenal blood flow was not altered during the renin infusion. During administration of the lower dose of renin (2.2 U as an initial injection, followed by in-

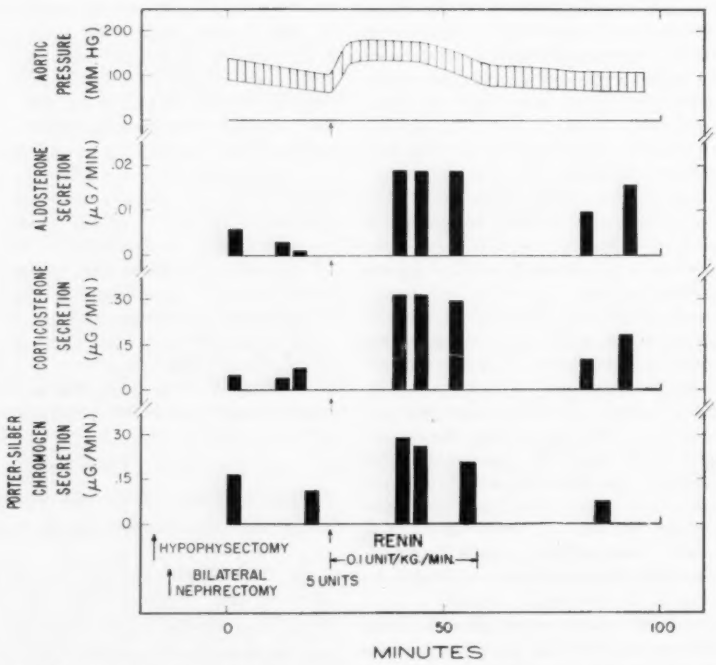

FIG. 1. EFFECTS OF RENIN ON ARTERIAL BLOOD PRESSURE, AND ON SECRETION RATES OF ALDOSTERONE, CORTICOSTERONE AND PORTER-SILBER CHROMOGENS IN A HYPOPHYSECTOMIZED-NEPHRECTOMIZED DOG.

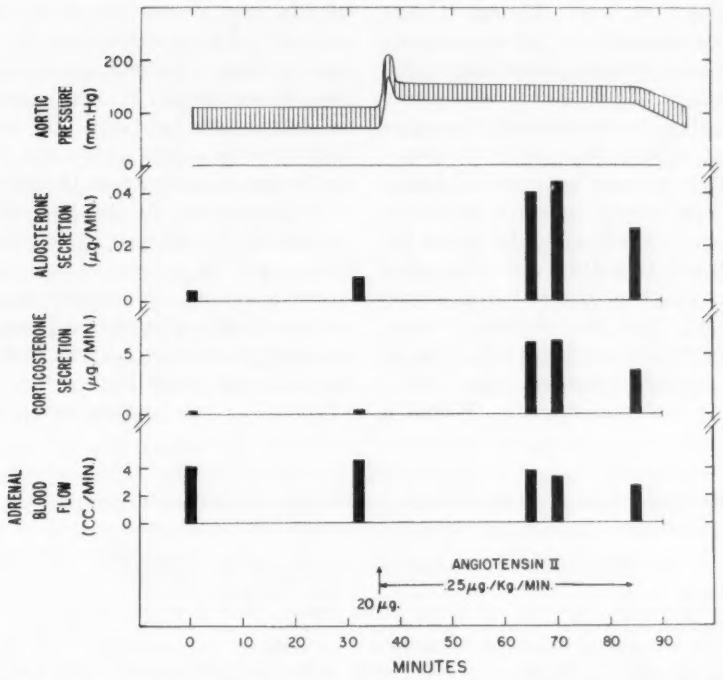

FIG. 2. EFFECTS OF ANGIOTENSIN II ON ARTERIAL BLOOD PRESSURE, ALDOSTERONE AND CORTICOSTERONE SERETION RATES AND ADRENAL BLOOD FLOW IN A HYPOPHYSECTOMIZED-NEPHRECTOMIZED DOG.

fusion at a rate of 0.03 U per kg per minute), aldosterone secretion increased in all 5 dogs, with an average increment of 172 per cent for the group; corticosterone output increased in 4 of the 5 dogs, with an average 316 per cent rise; and Porter-Silber chromogens increased in 3 of the 5 dogs, with an average increment of 67 per cent. A slight increase in adrenal blood flow was observed in 3 of the 5 dogs during the renin infusion. Secretion rates of each of the steroids had decreased, but had not returned to control levels, within 30 minutes after completion of the renin infusion in each of the 5 animals. In each animal the injection of renin resulted in a marked rise in mean arterial pressure, with an average increase of 50 mm Hg for the group of 5 dogs; the blood pressure remained at the elevated level throughout the period of renin infusion (Table I). After completion of the renin infusion, mean arterial pressure gradually returned to control levels over a 15 to 30 minute period.

Effects of infusion of angiotensin II at three dosage levels

A. High dosage level; 20 μg initially, followed by infusion at a rate of 0.25 μg per kg per minute into hypophysectomized-nephrectomized dogs. Increments in secretion of each of the steroids occurred, and marked increases in mean arterial pressure were observed in all 4 dogs. The average aldosterone output for the group increased from 0.004 to 0.021 μg per minute, a 425 per cent increment (Figure 2 and Dogs 1–4 of Table II). The average corticosterone secretion rate showed a 33-fold rise (Figure 2 and Table II). The average Porter-Silber chromogen production increased 375 per cent (Table II). Although the absolute increase was not so great for aldosterone as for corticosterone, the average level of aldosterone secretion during angiotensin II infusion was essentially the same as that observed in normal dogs stressed by laparotomy (17), whereas the experimental values for corticosterone (with

one exception, Dog 2 of Table II) and Porter-Silber chromogens remained far below the average values observed in stressed normal dogs (17). In the two animals in which adrenal effluent was collected at intervals after completion of the angiotensin II infusion, a sharp decrease in the secretion rates of all three steroids was observed within 30 minutes. Mean arterial pressure reached a maximum 30 to 45 seconds after the initial injection of angiotensin II and thereafter showed a gradual decline throughout the period of angiotensin II infusion. This slow decline in blood pressure probably reflects the progressive loss of vigor in these hypophysectomized dogs which were subjected to extensive surgery. Within 5 minutes after completion of the infusion, blood pressure had fallen below the initial control levels in all animals. Adrenal blood flow was not altered by angiotensin II; for the group of 4 dogs, the average adrenal blood flow was 3.4 ml per minute for the control period and 3.3 ml per minute during the angiotensin II infusion.

B. Intermediate dosage level; injection of 4 μg angiotensin II, followed by infusion at a rate of 0.05 μg per kg per minute into simple-hypophysectomized dogs. Aldosterone output increased in 3 of the 4 dogs, corticosterone production was augmented in all animals, and the Porter-Silber chromogen secretion rate increased in 3 of the 4 dogs (Table II). The average increment in secretion

TABLE II

Effects of angiotension II on adrenocortical steroid secretion and arterial blood pressure in dogs

Dog	Type of preparation*	Quantity of angiotensin II injected		Aldosterone secretion†		Corticosterone secretion†		Porter-Silber chromogen secretion		Mean arterial blood pressure‡		
		Start	Infusion	Before	During	Before	During	Before	During	Before	30 sec after	During infusion
		μg	μg/kg/min	μg/min		μg/min		μg/min		mm Hg		
1	H-N	20	0.250	0.001	0.018	0.01	0.09	0.03	0.11	75	125	120
2	H-N	20	.250	.006	.010	.04	1.78	.07	.71	100	140	136
3	H-N	20	.250	.001	.015	.01	.17	.12	.17	125	205	140
4	H-N	20	.250	.007	.042	.01	.62	.01	.17	80	180	145
Avg. 1–4		20	.250	.004	.021	.02	.67	.06	.29	95	163	135
5	S-H	4	.050	.002	.012	.05	.23	.02	.09	50	100	50
6	S-H	4	.050	.001	.007	.02	.12	.05	.06	90	155	115
7	S-H	4	.050	.006	.008	.37	.64	.23	.26	105	130	123
8	S-H	4	.050	.012	.024	.03	.16	.02	.10	85	132	108
Avg. 5–8		4	.050	.005	.013	.12	.29	.08	.13	83	129	99
9	S-H	0	.025	.014	.019	.25	.68	.07	.31	120		120
10	S-H	0	.021	.002	.007	.07	.09	.01	.01	82		82
11	S-H	0	.013	.004	.009	.01	.07	.01	.01	75		73
12	S-H	0	.013	.003	.003	.01	.01	.01	.01	126		136
13	S-H	0	.013	.030	.022	.11	.19	.10	.12	118		118
14	S-H	0	.013	.003	.003	.14	.30	.04	.11	132		124
15	S-H	0	.013	.004	.006	.19	.29	.05	.16	124		134
16	S-H	0	.013	.007	.013	.02	.04	.01	.03	105		108
17	S-H	0	.008	.003	.026	.04	.26	.11	.10	105		100
Avg. 9–17		0	.015	.008	.011	.09	.21	.05	.10	110		111
18	low Na-H-N	0	.013	.010	.007	.01	.02	.08	.06	96		95
19	low Na-H-N	0	.013	.007	.008	.01	.01	.11	.04	158		166
20	low Na-H-N	0	.008	.011	.013	.01	.01	.03	.05	96		110
Avg. 18–20		0	.011	.009	.009	.01	.01	.07	.05	117		124

* H-N, hypophysectomized-nephrectomized preparation; S-H, simple-hypophysectomized dog; low Na-H-N indicates animals which had been on a Na-free diet for 2 weeks, had been hypophysectomized for 3 days, and underwent bilateral nephrectomy 1 hour prior to the present acute study.

† Each value for aldosterone or corticosterone secretion represents the average of 2 or 3 determinations performed on separate samples of adrenal venous blood.

‡ "30 sec after" refers to the maximal mean arterial pressure 30 seconds after the starting dose of angiotensin II. "During infusion" indicates the average mean arterial pressure for the 40-minute period during which the angiotensin II was infused.

rate of each of the steroids (160 per cent for aldosterone, 142 per cent for corticosterone, 50 per cent for Porter-Silber chromogens) was less than that observed after the larger dose of angiotensin II was given to nephrectomized-hypophysectomized dogs; however, the reduction in increment in aldosterone secretion was not nearly so great as the decrease in increment in corticosterone output, and was also less than the reduction in increment in Porter-Silber chromogen production (Table II). The increases in steroid secretion rates during administration of angiotensin II at this dosage level were comparable in magnitude with the increases observed during infusion of renin at the rate described above. In the two animals in which postinfusion studies were made, secretion rates of aldosterone and corticosterone had decreased but had not returned to control levels within 20 minutes after completion of the angiotensin II infusion. Infusion of angiotensin II at this dosage level resulted in increases in mean arterial pressure in all animals, but the initial increases were not so marked as during administration of the larger dose to hypophysectomized-nephrectomized dogs, and the elevations of arterial pressure were not maintained throughout the period of study (Dogs 5–8, Table II). Adrenal blood flow showed no consistent changes during the angiotensin II infusion.

C. Low dosage level; infusion of angiotensin II at 0.008 to 0.025 μg per kg per minute. When angiotensin II was infused into 9 simple-hypophysectomized dogs at a rate slightly less than that which caused the minimal detectable increase in mean arterial pressure (no initial injection of angiotensin II was given) variable results were obtained (Dogs 9–17, Table II). Both dogs which received infusions at a rate of 0.025 and 0.021 μg per kg per minute showed increases in aldosterone secretion rates (Dogs 9 and 10, Table II). Of the 7 dogs given infusions at rates of 0.008 to 0.013 μg per kg per minute, three (Dogs 11, 16 and 17) showed an increase in aldosterone, while 5 animals (11, 13, 14, 15 and 17, Table II) responded with increases in corticosterone output; definite increases in Porter-Silber chromogen production occurred in 2 of the 7 dogs (14 and 15, Table II). However, it is noteworthy that the animal (Dog 17, Table II), which received angiotensin II at the lowest rate of infusion, showed unequivocal increases in both aldosterone and corticosterone production. For the group of 7 dogs given angiotensin II at rates of 0.008 to 0.013 μg per kg per minute, the increase in aldosterone production was not statistically significant (t, 1.32; $p > 0.2$), while a significant increase in the secretion rate of corticosterone (t, 3.13; $p < 0.02$) occurred. No significant change in Porter-Silber chromogen output was observed at this dosage level (t, 1.74; $p > 0.1$). Mean arterial blood pressure remained essentially unchanged (t, 0.37; $p > 0.9$) during the angiotensin II infusion in these animals. Adrenal blood flow showed little variation during the period of study in any of the 9 dogs in this group.

Infusion of angiotensin II at rates of 0.008 to 0.013 μg per kg per minute into 3 hypophysectomized-nephrectomized dogs, pretreated by 2 weeks on a Na-free diet, did not affect the low basal rates of secretion of aldosterone, corticosterone or Porter-Silber chromogens in any animal (Dogs 18–20, Table II). It should be emphasized that all values for aldosterone and corticosterone secretion represent the average of two or three determinations from separate collections of adrenal vein blood.

Aldosterone secretion in experimental renal hypertension

An increase in mean arterial pressure was observed in every dog within 1 week after the left (first) Goldblatt clamp had been placed; the average increase in mean arterial pressure at this time was 26 mm Hg, with a range of 10 to 47. In each dog a further increase in arterial pressure was observed after the application of the second Goldblatt clamp. The behavior of the dogs after constriction of the right (second) renal artery followed one of two general patterns. In the first group of dogs (1–8, Table III), each animal, despite the development of sustained moderate to severe hypertension, continued to appear healthy and to eat well (with one exception, Dog 6 of Tables III and V, which is discussed below), and all animals survived until they were sacrificed to obtain the kidneys for renin assays; the group of dogs was considered to have benign renal hypertension. The second group (Dogs 9–15, Table IV) exhibited progressive weakness, lethargy, anorexia and preterminal vomiting, and died from

TABLE III

Adrenocortical steroid secretion in dogs with experimental benign renal hypertension

Dog	Aldosterone secretion	Corticosterone secretion	Avg. mean arterial pressure*		Duration, bilat. renal art. constrict.†
			Control	Exp.	
	μg/min	*μg/min*	*mm Hg*		*days*
1	0.012	1.70	137	220	4
2	.018	1.62	118	180	3
3	.042	3.80	133	199	12
4	.028	2.34	133	180	13
5	.011	1.28	124	185	24
6	.065	3.07	121	192	4
7	.021	3.29	131	187	4
8	.023	2.82	128	190	6
Average	.028	2.49	129	192	9
Normal dogs stressed by laparotomy n = 26	0.024 ±0.017	2.34 ±0.84			

* Values represent the average mean arterial pressures during the 4-week period after application of the second Goldblatt clamp.

† Number of days after second Goldblatt clamp was placed. The first Goldblatt clamp was placed 1 to 2 weeks before the second.

7 to 21 days after constriction of the second renal artery; this group of animals was considered to have malignant renal hypertension. These two groups will be considered separately.

For the dogs with benign hypertension, the average mean arterial pressure during the 4-week period following constriction of the second renal artery was 192 mm Hg, representing a 49 per cent increase over the average control value. Serum Na and K concentrations remained essentially unchanged after the development of hypertension in the 3 animals in which electrolyte determinations were made. At the time of laparotomy the aldosterone secretion rates ranged from 0.011 to 0.065 μg per minute; in only one (Dog 6 of Table III) did the aldosterone output significantly exceed the average value observed in normal dogs stressed by laparotomy. The average value for the group of 8 dogs was 0.028 μg per minute (Table III); this value does not differ

TABLE IV

Adrenocortical steroid secretion in dogs with experimental malignant renal hypertension

Dog	Aldostrone secretion	Corticosterone secretion	Avg. mean arterial pressure*		Duration, bilat. renal art. constrict.†
			Control	Exp.	
	μg/min	*μg/min*	*mm Hg*		*days*
9	0.047	2.95	115	212	19
10	.053	2.26	125	200	6
11	.041	2.43	134	212	4
12	.044	0.29	118	188	3
13	.066	1.78	121	199	3
14	.043	4.19	132	184	6
15	.168	2.07	124	203	3
Average	.066	2.28	124	200	6
Normal dogs stressed by laparotomy n = 26	0.024 ±0.017	2.34 ±0.84			

* Values represent the maximal arterial pressures observed prior to the animals' demise.

† Number of days after second Goldblatt clamp was placed. The first Goldblatt clamp was placed 1 to 2 weeks before the second.

TABLE V

Aldosterone and corticosterone secretion rates in dogs with experimental renal hypertension

Dog	Steroid secret.*	At laparotomy	During collections from conscious, unstressed animals via chronic adrenal venous catheter. Days after adrenal venous catheter placed												Avg. values, conscious unstressed dogs
	μg/min		*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	
Benign hypertension															
1	A	0.012	0.009	0.006		0.003	0.015	0.008	0.011	0.005			0.002		0.007
	C	1.70	0.09	0.06		0.17	2.36	0.16	0.80	0.12			0.27		0.50
2	A	0.018		0.007				0.006	0.001	0.003	0.024				0.008
	C	1.62		1.85				0.21	0.74	0.49	0.42				0.74
5	A	0.011		0.005	0.007		0.003		0.003		0.003				0.004
	C	1.28		0.71	0.17		0.07		0.11		0.08				0.23
6	A	0.065				0.026	0.032	0.019	0.016		0.003	0.010	0.007	0.009	0.015
	C	3.07				0.34	0.75	0.14	0.50		0.33	0.50	0.46	1.56	0.70
7	A	0.021		0.004	0.001		0.001	0.004							0.003
	C	3.29		1.26	0.12		0.13	0.38							0.47
8	A	0.023		0.003	0.003		0.006	0.005		0.004					0.004
	C	2.82		0.21	0.09		0.08	0.12		0.62					0.22
Average	A														0.007
	C														0.48
Malignant hypertension															
11	A	0.041				0.024	0.036	0.055†							0.038
	C	2.43				1.00	0.44	0.39							0.61
13	A	0.066	0.085	0.073†											0.079
	C	1.78	1.40	1.07											1.24
14	A	0.043	0.075	0.074		0.080†									0.076
	C	4.19	2.17	0.84		0.41									1.14
15	A	0.168		0.087†											0.087
	C	2.07		0.65											0.65
Average	A														0.070
	C														0.91
Normal dogs															
Normal Dogs	A	0.024 ±0.017													0.006 ±0.002
	C	2.34 ±0.84 n = 26													0.29 ±0.20 n = 5

* A, aldosterone; C, corticosterone.
† All dogs in the malignant hypertensive group died between 12 and 24 hours after the final collections of adrenal venous blood were made.

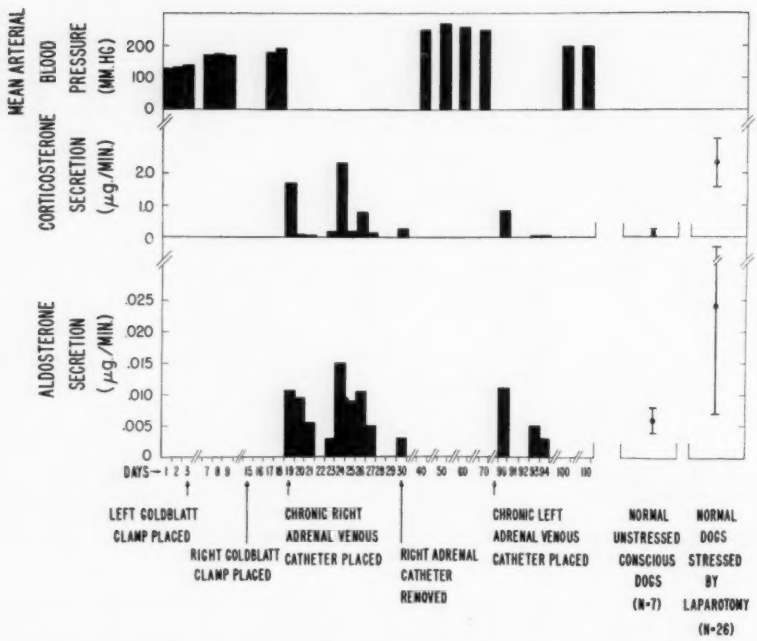
MEAN ARTERIAL BLOOD PRESSURE (MM HG)
200
100
0
CORTICOSTERONE SECRETION (μg/MIN.)
20
10
0
ALDOSTERONE SECRETION (μg/MIN.)
.025
.020
.015
.010
.005
0
DAYS— 1 2 3 7 8 9 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 40 50 60 70 90 91 92 93 94 100 110
LEFT GOLDBLATT CLAMP PLACED
RIGHT GOLDBLATT CLAMP PLACED
CHRONIC RIGHT ADRENAL VENOUS CATHETER PLACED
RIGHT ADRENAL CATHETER REMOVED
CHRONIC LEFT ADRENAL VENOUS CATHETER PLACED
NORMAL UNSTRESSED CONSCIOUS DOGS (N=7)
NORMAL DOGS STRESSED BY LAPAROTOMY (N=26)

FIG. 3. ALDOSTERONE AND CORTICOSTERONE SECRETION RATES AND MEAN ARTERIAL PRESSURE IN A DOG WITH BENIGN RENAL HYPERTENSION. Note that the average rate of aldosterone secretion is essentially the same as that observed in normal conscious dogs, and that the aldosterone output exceeds 0.010 μg per minute only on those days on which the simultaneous corticosterone secretion rates are elevated above the basal level (Dog 1 of Tables III and V).

significantly from the average aldosterone secretion rate of 0.024 ± 0.017 μg per minute SD observed in 26 normal dogs stressed by laparotomy (t, 0.57; $p > 0.5$). The average corticosterone secretion rate for the group of 8 dogs at the time of laparotomy was 2.49 μg per minute, which is essentially the same as the average value of 2.34 $\pm$ 0.84 μg per minute SD observed in 26 normal dogs stressed by laparotomy (t, 0.57; $p > 0.5$). The mean adrenal blood flow of 4.0 ml per minute was greater than the average value of 2.9 for normal stressed dogs, but the difference is not statistically significant (t, 1.93; $p > 0.05$).

In 6 dogs of this group (1, 2, 5–8, Tables III and V) adrenal venous blood was collected at intervals over a 7 to 12 day period, via an indwelling adrenal venous catheter, while the animals were in a conscious, unstressed state. In these animals the average aldosterone secretion rate was 0.007 μg per minute, a value that agrees closely with the average output of 0.006 ± 0.002 μg per minute reported for normal, unstressed conscious dogs (24). The average corticosterone secretion rate for this same period was 0.48 μg per minute, which is not significantly different from the value of 0.29 ± 0.20 μg per minute SD observed in normal unstressed, conscious dogs (t, 1.42; $p > 0.1$). In the dog (Figure 3, Dog 1 of Tables III and V) in which subsequent secretion studies were made 3 months after application of the second Goldblatt clamp by placing an indwelling catheter in the left adrenolumbar vein, the average secretion rates for aldosterone and corticosterone were 0.007 μg per minute and 0.29 μg per minute, respectively. In the one animal in which the aldosterone secretion rate during laparotomy appeared to be significantly elevated (Dog 6 of Tables III and V), the aldosterone output remained quite high during the first week after placement of the indwelling adrenal catheter, but was consistently within normal limits during the second week of studies in the conscious animal; the corticosterone

secretion rate showed no significant change during this time (Table V). In the remaining 5 dogs of this group the aldosterone secretion rate exceeded 0.010 μg per minute on only four occasions (Day 9 for Dog 2 of Table V, and Days 5, 7 and 72 after the catheter was placed in Dog 1 of Table V and Figure 3). On two of the four days on which the aldosterone output exceeded 0.010 μg per minute, the simultaneous corticosterone secretion rate was greater than 1.80 μg per minute (Figure 3); these very high values for corticosterone output probably reflect a greater than basal rate of release of ACTH. It is suggested that the slightly higher aldosterone secretion rates on these days were secondary to increased ACTH release and did not result from increased secretion of the renal aldosterone-stimulating substance. The rate of corticosterone secretion during infusion of angiotensin II was considerably less than 1.80 μg per minute during aldosterone secretion rates of the magnitude (0.010 to 0.015 μg per minute) observed here.

For the group of 7 dogs (9–15, Table IV) with malignant hypertension, the blood pressure increased more rapidly after constriction of the right (second) renal artery. The average of the maximal observed mean arterial pressures for this group of dogs was 200 mm Hg, representing a 61 per cent increase over the control value. No significant changes in serum Na and K levels were observed after the development of hypertension in any of the four animals in which electrolyte determinations were made. The explanation for this finding is not clear in view of the striking hyperaldosteronism present. At the time of laparotomy, aldosterone secretion rates ranged from 0.041 to 0.168 μg per minute, with a mean aldosterone secretion rate of 0.024 ± 0.017 μg per minute SD observed in normal dogs stressed by laparotomy (t, 2.5; $p < 0.02$). The average corticosterone secretion rate was 2.28 μg per minute, which is essentially the same as the value of 2.34 ± 0.84 μg per minute SD observed in normal dogs stressed by laparotomy.

It is noteworthy that in the two dogs in which the highest aldosterone secretion rates were ob-

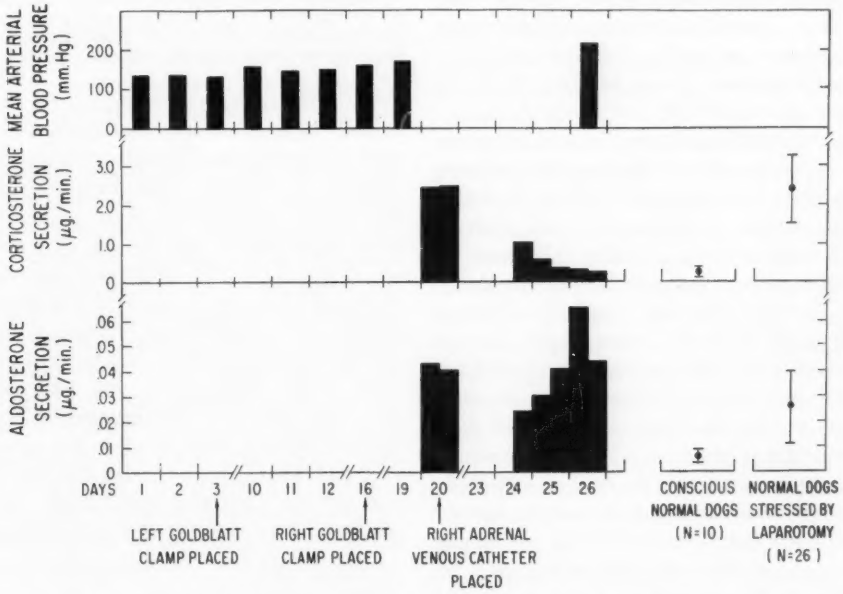

FIG. 4. ALDOSTERONE AND CORTICOSTERONE SECRETION RATES AND MEAN ARTERIAL PRESSURES IN A DOG IN THE MALIGNANT PHASE OF EXPERIMENTAL RENAL HYPERTENSION. Note the marked hypersecretion of aldosterone during the last two days of the study, at a time when corticosterone production has decreased to the basal level observed in conscious normal dogs (Dog 11 of Tables IV and V).

served (Dogs 13 and 15 of Table IV), approximately 500 ml of ascitic fluid was present at the time of adrenolumbar vein cannulation. A similar observation has subsequently been made in one additional animal with malignant renal hypertension. No ascites was present in either of the first two animals at the time of autopsy 3 days after laparotomy.

In the 4 dogs of the malignant hypertensive group in which adrenal effluent was collected for 3 to 8 days from the conscious animals via the indwelling adrenal venous catheter, the average aldosterone secretion rate was 0.070 μg per minute. If the days on which corticosterone secretion exceeded 0.65 μg per minute (i.e., the early postoperative days on which ACTH release greatly exceeded basal levels, presumably as a result of the stress of laparotomy) are excluded, the average aldosterone secretion rate was 0.067 μg per minute, which represents a greater than 10-fold increase over the average value of 0.006 μg per minute observed in normal unstressed, conscious dogs. Figure 4 presents data on the malignant hypertensive dog from which adrenal venous samples were collected for the longest period of time (Dog 11, Table IV). Note that at the time of laparotomy the corticosterone as well as the aldosterone secretion rate was elevated as a result of increased ACTH release secondary to the stress of surgery. However, during the last three days of collection, at a time when the animal was conscious, afebrile and unstressed, corticosterone output showed a progressive decline to the basal levels observed in normal conscious dogs; during this same period, aldosterone secretion rate, which was always at least four times the value observed in normal conscious dogs, showed a progressive increase to a maximal value eight times that observed in unstressed normal dogs. Similar observations were made in Dog 14 (Table V) in which aldosterone secretion progressively increased to a value of 0.080 μg per minute on the final day of study, at which time the corticosterone output had decreased to a low basal level of 0.41 μg per minute. The presence of this very high aldosterone secretion rate, in the presence of a low basal corticosterone output, indicates that the observed increment in aldosterone secretion was not dependent on increased ACTH release.

The average renin content (the product of the renin yield per gram of tissue and the total weight of both kidneys) of the two kidneys of the dogs with malignant renal hypertension was 160 dog U (range, 125 to 185), while the average renin content of the two kidneys of the dogs with benign renal hypertension was 29 dog U, (range, 19 to 38; Table VI). The difference between the benign and malignant hypertensive groups is highly significant statistically (t, 7.37; $p < 0.01$). The average renin content of the kidneys of the dogs with benign hypertension was also greater than the renin content of the kidneys of normal dogs which had been maintained on the same synthetic diet and electrolyte intake for 5 to 10 days before the kidneys were removed (Table VI), but the difference was of borderline statistical significance (t, 2.66; $0.05 > p > 0.02$).

TABLE VI

Aldosterone secretion rates and renin content of kidneys of dogs with benign and malignant hypertension

Dog	Aldosterone secretion: At laparotomy	Aldosterone secretion: Via indwelling catheter in conscious, unstressed dogs	Renin content, kidneys
	μg/min		dog U/2 kidneys
Benign hypertension			
1	0.012	0.007	19
2	.021	.002	38
8	.023	.004	30
X*			28
Average	.019	.004	29
Malignant hypertension			
12	.044		185
13	.066	.079	178
14	.043	.076	153
15	.168	.087	125
Average	.080	.081	160
Normal dogs			
Average ± SD	.024 ±.017 n = 26	.006 ±.002 n = 5	15 ±7.1 n = 7

* Dogs are numbered as in Tables III, IV, and V, with the exception of Dog X which was an animal with benign renal hypertension of 2 months' duration, on which steroid secretion rates were not determined.

DISCUSSION

The concept that aldosterone secretion is regulated by the renin-angiotensin system is derived from the findings of many investigators (1–14).

This hypothesis was suggested by the indirect evidence that renin injections cause hypertrophy of the adrenal zona glomerulosa (6) and that the degree of granulation of the juxtaglomerular (JG) cells is directly correlated both with the amount of extractable renin in the kidney (9, 10, 13) and with the width of the adrenal zona glomerulosa (7, 8, 14). It has recently received strong support from the direct demonstration that an aldosterone-stimulating factor is secreted by the kidney (1–3) and that aldosterone-stimulating activity is confined to the renin fraction of crude kidney extracts (4). Tobian, after clearly summarizing the findings of many investigators in this field, (13, 14), has further postulated that the cells that produce renin, the JG cells (25, 26), located in the media of the renal afferent arterioles, act as baroreceptors responsive to the degree of filling of these vessels (13, 27). On the basis of this hypothesis, the JG cells would respond to decreased stretch of the renal afferent arterioles by an increase in renin secretion with a consequent augmentation of aldosterone output, and vice versa. Thus, the renin-angiotensin system, which appears to serve as a homeostatic mechanism in the maintenance of arterial blood pressure (28), could also perform a homeostatic function in the control of aldosterone secretion. The present experimental results are consistent with this concept.

The finding that infusion of very small quantities of renin increased the secretion rate of aldosterone in the hypophysectomized-nephrectomized dog adds support to the theory that the renin-angiotensin system performs a physiological role in the control of aldosterone secretion. Although renin infusion in the present experiments resulted in increased production of corticosterone and Porter-Silber chromogens as well as aldosterone, the pattern of response was quite different from that seen during infusion of ACTH (29). Renin infusion increased the rate of aldosterone secretion to levels similar to those observed during infusion of ACTH in acutely hypophysectomized dogs (29) and after the stress of laparotomy in normal dogs (17). In marked contrast to these findings, the values during renin infusion for corticosterone and Porter-Silber chromogen output were approximately 10 and 2 per cent, respectively, of the values observed in normal dogs stressed by laparotomy (17, 24) and in hypophysectomized dogs given ACTH infusions (29). In fact, the secretion rates during renin infusion for corticosterone and Porter-Silber chromogens are essentially the same as the values observed in conscious, unstressed normal dogs with chronic adrenal venous catheters (24). Since it has been demonstrated (24) in studies on conscious dogs that corticosterone and Porter-Silber chromogen secretion rates may be low in the presence of a very high rate of aldosterone production (and presumably in the presence of increased quantities of the aldosterone-stimulating factor produced by the kidney), the results from the present renin infusion experiments are consistent with the concept that the renal aldosterone-stimulating factor is identical with renin.

The demonstration that angiotensin II also increases aldosterone output is in agreement with the previously reported findings of both Biron and associates (12) and Laragh, Angers, Kelly and Lieberman (11) that angiotensin II infusion consistently increases aldosterone production in man. The present results, obtained from hypophysectomized dogs, clearly demonstrate that angiotensin II increases aldosterone secretion by a non-ACTH mechanism, a finding that was suggested by the pattern of steroid secretion observed in the earlier studies in man (11, 12). The very marked increase in corticosterone observed in two of the four dogs which received the largest dose of angiotensin II would at first appear inconsistent with the hypothesis that the renin-angiotensin system regulates aldosterone secretion, since a maximal rate of aldosterone secretion is frequently observed in the presence of a very low basal corticosterone output in experimental secondary hyperaldosteronism (24). However, the rate of infusion of angiotensin II at the highest dosage level almost certainly exceeds the rate at which angiotensin II is released under physiological conditions (30). It is entirely possible that very great increases in activity of the renin-angiotensin system may cause appreciable increases in corticosterone as well as aldosterone. Indeed, one recent study (3) has demonstrated that nephrectomy of both hypophysectomized caval and hypophysectomized Na-depleted dogs resulted in a marked decrease in corticosterone as well as aldosterone secretion, and that infusion of extracts of renal tissue from dogs

with secondary hyperaldosteronism increased both corticosterone and aldosterone production.

The finding that the intermediate dosage of angiotensin II (the pressor effect of which was roughly equivalent to the pressor effect of the renin used in Experiment I) caused essentially the same qualitative changes in steroid secretion as did the renin infusion, suggests that renin exerts its effect on aldosterone secretion via angiotensin II. It is noteworthy that the increments in corticosterone (2- to 6-fold) and Porter-Silber chromogen (0- to 4-fold) production observed during infusion of the intermediate dosage of angiotensin II are roughly comparable with the increments in cortisol and tetrahydrocortisol excretion (2- to 3-fold) noted by Genest (31) during the infusion of angiotensin II in man at rates that caused moderate, sustained elevations of blood pressure. As pointed out by this investigator, increases in this magnitude of 17-hydroxycorticoids and of corticosterone are "small in terms of physiological significance" in comparison with the concomitant increases in aldosterone output that occurred during the angiotensin II infusion.

The results obtained after the infusion of 0.008 to 0.025 μg per kg per minute of angiotensin II indicate that the minimal effective steroid-stimulating dose of angiotensin II may vary appreciably from animal to animal. Responses in aldosterone secretion occurred in the absence of an increase in blood pressure but never without a slight concomitant increase in corticosterone output. It has been reported by Genest (31), in regard to the response to angiotensin II infusions in man, that the minimal effective dose for blood pressure response exceeds the dose necessary to produce a measurable increase in aldosterone production. Although the present experimental data suggest that this situation may prevail in the dog, the present findings, obtained from anesthetized dogs which were hypophysectomized or hypophysectomized and nephrectomized, cannot be extrapolated with certainty to the conscious intact animal. Indeed, Goldblatt (32) has pointed out that the blood pressure response to renin in the barbiturate-anesthetized dog is markedly decreased as compared with the blood pressure response in normal conscious dogs.

Since the results from infusion of renin and angiotensin II support the hypothesis that aldosterone secretion is regulated by the renin-angiotensin system, the question arises as to the nature of the stimulus to renin production. Goldblatt, in his classic series of studies (32) showed that the renin content of the kidneys increased after bilateral renal arterial constriction; it was then postulated that diminished renal blood flow (RBF) was the immediate stimulus to renin production. Corcoran and Page (33) subsequently showed that diminished renal blood flow was not invariably present in animals with experimental renal hypertension, and suggested that diminished pulse pressure in the renal arterioles provided the stimulus to renin production in these circumstances. However, later evidence (34) indicated that in animals with experimental renal hypertension secondary to encapsulation of the kidney, the intrarenal pulse pressure was increased rather than decreased. More recently Tobian (13) has pointed out that decreased stretch in the renal afferent arterioles (resulting in the clamped kidney from decreased renal arteriolar pressure, and in the encapsulated kidney from increased extrinsic pressure from the tissues surrounding the afferent arteriole) may be a common factor to both types of renal hypertension, and has postulated that decreased stretch provides the stimulus to increased renin production by the juxtaglomerular cells in both situations. This suggestion appears consistent with available data.

In the present experiments aldosterone secretion was increased above the normal range in only one of eight dogs with benign renal hypertension. This finding appears inconsistent with the concept that aldosterone secretion is regulated by the renin-angiotensin system, since earlier experiments indicated that increased production of renin is present in the majority of animals with renal hypertension during the first 3 months after bilateral renal arterial constriction (16, 32, 35). Two explanations for this apparent inconsistency present themselves. In the first place, it is possible that increased renin secretion does not occur in the benign phase of experimental renal hypertension. Although increased renin *content* of the kidneys has appeared consistently to be present in previous studies of benign experimental and human essential hypertension (16, 32), increased renin *secretion* by the kidney in these situations has not been conclusively demonstrated in either

man or dog (13, 36, 37). In the present dogs with benign experimental renal hypertension, the renin content of the kidneys, although greater that that found in normal dogs, was considerably lower than that observed in malignant renal hypertension. It is possible in benign experimental hypertension either that the hypertension is caused by increased sensitivity to small amounts of renin or that the hypertension is not solely dependent upon the renin-angiotensin system. In the second place, it is possible that the amount of renin secreted by the dogs with benign experimental hypertension, although increased to a level adequate to produce and maintain hypertension, is not adequate to cause hypersecretion of aldosterone. This latter alternative seems less likely in view of the previously cited findings of Genest (31) that angiotensin II infusion at a rate too low to elicit a blood pressure response augmented aldosterone output in man.

In the present study the dog in the benign hypertensive group in which the greatest rate of aldosterone production was noted (Dog 6 of Tables III and V) deserves special comment. This dog, alone among the animals in the benign group, appeared clinically to be developing malignant hypertension; beginning 3 days after the second Goldblatt clamp was placed (one day before the chronic adrenal catheter was inserted), he showed progressive lethargy, anorexia (but no vomiting despite forced feeding of diet) and weight loss. These symptoms persisted for 7 to 8 days, after which gradual improvement was observed. The symptoms coincided with the period during which hypersecretion of aldosterone was observed in this dog (during laparotomy and on Days 1–7 of Table V). It appears possible that both the malignant hypertensive symptoms and the hypersecretion of aldosterone were reflections of a marked increase in renin secretion resulting from a very substantial reduction in pressure in, or flow through, the renal afferent arterioles; and that both the symptomatic improvement and the decrease in aldosterone output resulted from the development of collateral renal arterial blood supply with a consequent decrease in renin production. In the present experiments extensive renal collateral vessels were observed within 10 days after application of the second Goldblatt clamp, and earlier studies (38) have demonstrated that renal blood flow may return to normal prehypertensive levels within 2 to 3 weeks after moderately severe constriction of the renal artery of a single explanted kidney.

The finding of consistent increases in aldosterone secretion rate in dogs with malignant hypertension is in agreement with the similar findings in man by Genest (39), Laragh (40) and Venning (41), and their co-workers and is entirely consonant with the hypothesis that the renin-angiotensin system performs a major role in the regulation of aldosterone secretion. The majority of reports indicate that renin secretion by the kidneys, as well as renin content of the kidneys, is increased in the malignant phase of both human essential and experimental renal hypertension (13, 30, 35, 42). This hypothesis is further supported by the present experimental finding that the renin content of the kidneys of the malignant hypertensive dogs, which exhibited marked hypersecretion of aldosterone, was greatly increased over the renin content in the benign hypertensive dogs in which aldosterone production was only slightly above the normal range.

Since the present experimental data are consistent with the hypothesis that aldosterone secretion is regulated by the renin-angiotensin system, the question arises as to why hypertension is not present in such situations as congestive heart failure, the post-hemorrhagic state, during thoracic inferior vena caval constriction, and during chronic Na depletion—conditions in which hypersecretion of aldosterone is a consistent finding (1, 3, 43). Increased renin secretion has been reported in congestive heart failure (44) and after hemorrhage (45); and hyperplasia and hypergranulation of the juxtaglomerular cells, which provide presumptive evidence of increased renin production (14, 46), have recently been found after thoracic caval constriction (47) and during chronic Na depletion (9). The explanation may be that in these situations increased circulating angiotensin II, with a consequent increase in total peripheral resistance, serves to maintain a normal blood pressure. Thus, the renin-angiotensin system may act as a homeostatic mechanism (8, 28) to maintain an adequate flow of blood to vital organs under these circumstances. Recent observations that the blood pressure response to angiotensin II is greatly reduced in the

dog during chronic Na depletion or during thoracic caval constriction (48) support this view. Available data therefore appear to be uniformly consistent with the hypothesis that the renin-angiotensin system plays an important role in the regulation of aldosterone production in a variety of circumstances in which hypersecretion of aldosterone is known to occur.

SUMMARY AND CONCLUSIONS

Infusion of renin into hypophysectomized-nephrectomized dogs increased the rates of secretion of aldosterone, corticosterone and Porter-Silber chromogens by a non-ACTH mechanism. Infusion of synthetic angiotensin II into hypophysectomized-nephrectomized or simple-hypophysectomized dogs (only anterior pituitary removed) likewise augmented the production of each of the steroids measured; a decrease in the rate of infusion of angiotensin II reduced the increment in corticosterone secretion to a far greater extent than the increment in aldosterone secretion. With doses of angiotensin II too small to elicit a blood pressure response, physiologically significant increases in aldosterone secretion were sometimes observed. At this very low dosage level, the increments in corticosterone output were quantitatively slightly greater than those of aldosterone but were at a level which usually has no physiological effect, and no significant changes in Porter-Silber chromogen production occurred. The renin content of the kidneys of dogs with benign renal hypertension was only slightly increased, and aldosterone secretion was within normal limits in these animals. A 10-fold increase in the renin content of the kidneys was observed in dogs with malignant renal hypertension, and aldosterone output was markedly increased, by a non-ACTH mechanism, in this group of dogs. These data are consistent with the concept that the renin-angiotensin II system plays an important physiological role in the regulation of aldosterone secretion.

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THE EFFECT OF SALT DEPRIVATION ON THE URINARY CONCENTRATING MECHANISM IN THE DOG*

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According to the countercurrent theory (1–3), concentration of the urine results from the movement of water from the collecting tubule into the hypertonic medullary interstitium. Medullary hypertonicity is generated and maintained by the sodium transported out of the ascending limb of the loop of Henle and trapped in the medulla by the vasa recta which form a countercurrent exchanger. Consequently, the availability of sodium for reabsorption in the loop of Henle may assume a critical role in the regulation of urinary concentration.

Various derangements in water excretion have been detected when the intake or renal excretion of sodium is altered. Patients with congestive heart failure, cirrhosis of the liver, and other conditions characterized by diminished sodium excretion frequently have been reported to have impaired urinary concentration in the absence of intrinsic renal disease. Levinsky, Davidson and Berliner (4) noted that dogs maintained on a sodium-free diet showed decreased maximal urinary concentration. Levitt, Levy and Polimeros (5), however, found that salt restriction in normal human subjects for 5 days had no effect on maximal urinary concentration.

The present studies were undertaken to examine the relationship of sodium metabolism to urinary concentrating ability by evaluating the effects of varying sodium intake, glomerular filtration rate (GFR), aldosterone activity, and serum sodium concentration on renal water conservation in dogs, as reflected by their capacity to elaborate a maximally concentrated urine and to form T^cH_2O.

METHODS

Female mongrel dogs weighing 8 to 18 kg were used. Each dog was tube-fed once daily an artificial diet that provided 4.3 mEq potassium, 1.1 g protein, 27 calories, and 30 ml water per kg of body weight per day. When access to sodium was permitted, 1.5 mEq sodium per kg body weight per day was added to the diet, whereas the sodium-free diet provided less than 0.02 mEq sodium per kg body weight per day. The amount of diet fed any given dog was kept constant.

Acute experiments were performed to evaluate maximum urinary concentrating ability and the maximum rate of T^cH_2O formation. Food and water were withheld for the 24 hours preceding the experiment. Two hours prior to study, 5 U of vasopressin (Pitressin tannate in oil) was administered i.m. At the time of the study, dogs were lightly anesthetized with sodium pentobarbital, and bladder urine was obtained at once for the determination of maximum urinary concentration. To measure T^cH_2O formation, 10 per cent mannitol in 5 per cent dextrose in water was infused intravenously at a rate of approximately 1 ml per minute during the 20-minute equilibration period; subsequently the rate was increased in stepwise fashion to induce graded solute diuresis. In accordance with the suggestion of Giebisch and Lozano (6), 75 mmoles of sodium chloride was added to each L of mannitol infusion to minimize undue washout of papillary sodium by osmotic diuresis alone (7). To assure sustained maximal levels of antidiuretic hormone (ADH), 33 mU per kg body weight vasopressin was given with the inulin prime at the start of the equilibration period and thereafter constantly infused at a rate of 50 mU per kg per hour in 5 per cent dextrose in water, adjusted to pH 5.5 with acetic acid. Inulin was infused at a constant rate of 10 mg per minute. Urine was allowed to drain freely from an indwelling bladder catheter. Collection periods were terminated by air washout and manual compression of the bladder. Arterial blood samples were drawn at midpoints of the urine collection periods into heparinized syringes. Osmolalities of serum and urine were determined on the Fiske osmometer. Inulin was measured by the resorcinol method of Schreiner

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† Work done as a Public Health Service trainee of The National Institutes of Health.

(8). Sodium and potassium were determined with an internal standard flame photometer.

The effects of variations in salt intake, serum sodium concentration, and aldosterone on maximum urinary concentration and maximum T^cH_2O formation were investigated in dogs treated as follows.

A) Sodium-fed control. Dogs received sodium-containing diet for 2 to 5 days.

B) Sodium-restricted. Dogs received sodium-free diet for 3 to 9 days.

C) Aldosterone and aldosterone antagonists. Dogs receiving sodium-containing diet were given DL-aldosterone[1] in a dose of 30 to 500 μg per kg body weight i.v. 2 hours prior to study. Dogs receiving sodium-free diet for 1 week were then maintained on this diet and a spirolactone was given for an additional week. One dog was given SC-9420,[2] 100 mg orally once daily; the other five were given SC-11929,[3] 10, 20, or 100 mg i.m. once daily.

D) Raising GFR during sodium restriction. GFR was raised in sodium-restricted dogs by the administration of slowly released methylprednisolone (Depo-Medrol), 50 mg i.m., every other day for 4 to 6 days, and regular methylprednisolone (Solu-Medrol), 40 mg i.v., 20 minutes prior to onset of the study. One dog was given only the acute dose.

E) Hyponatremia. Hyponatremia was induced by the administration of 5 U of vasopressin i.m. at 7 a.m. and 4 p.m. daily. These dogs received either the sodium-free or sodium-containing diet as indicated and were tube-fed additional water to make a total of 4 to 6 per cent of body weight per day. Studies were done in 3 to 4 days depending on the degree of hyponatremia present.

At least 3 days elapsed between consecutive studies in any given dog. Since obligatory sodium loss during diuresis in sodium-deprived dogs averaged 20 mEq, this amount of sodium was given to all dogs with the next feeding after study. Free access to water was allowed until 48 hours prior to study to prevent dehydration, and i.m. iron was given to prevent iron deficiency.

CALCULATIONS

For purposes of comparison, results are discussed in terms of the regression of osmolar clearance (C_{osm}) on flow in milliliters per minute (V). C_{osm} is defined as $U_{osm}/P_{osm} \times V$. T^cH_2O refers to water reabsorbed in excess of solute as measured from isosmotic urine, and is derived by the equation $T^cH_2O = C_{osm} - V$, after Zak, Brun and Smith (9). Slopes were obtained by the method of least squares from observations made at flows between 3 and 13 ml per minute in which range the regression is linear. Since the slope of regression may vary widely from unity, maximum T^cH_2O ($T^c_MH_2O$) in this report was estimated from the single greatest observed T^cH_2O corrected to 100 ml GFR [$T^c_MH_2O = (\max T^cH_2O/GFR) \times 100$] rather than the usual value that represents the average T^cH_2O over the linear range of the regression. Since the T^cH_2O formation was not constant under certain experimental conditions, an expression was devised to compare T^cH_2O at comparable urine flows corrected for variations in body size. This expression, referred to as $T^c_DH_2O$, was calculated as that value of T^cH_2O formed at a urine flow equal to one-half of the initial body weight in kilograms of a given dog and corrected to 100 ml GFR. The point at which the regression line of T^cH_2O intersected the isosmotic line was designated the crossover point, and represents the beginning of the formation of hypotonic urine. All statistical analyses were done by standard methods (10). The number of periods in each experiment varied from 4 to 15, the usual being 6 or 7. Collection periods usually lasted 10 minutes. Only studies in which GFR was relatively stable throughout are reported.

RESULTS

At the onset of this study, two sodium-fed trained dogs were studied supine and unanesthetized. Several days later they were restudied after being anesthetized in the manner described for all subsequent studies. In neither dog did anes-

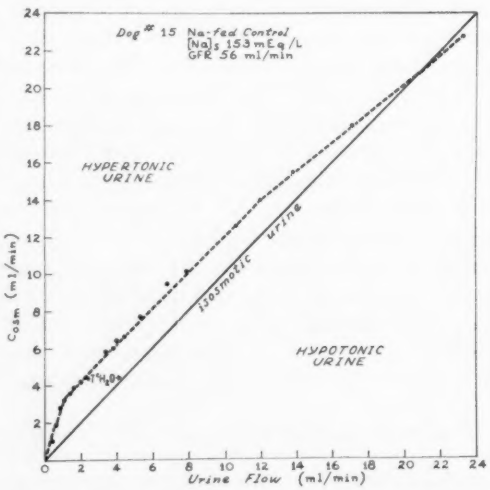

FIG. 1. THE FORMATION OF T^cH_2O DURING MANNITOL DIURESIS IN A SODIUM-FED CONTROL DOG. In this and subsequent figures, C_{osm} and urine flow were not corrected to a standard GFR. Therefore, the values for T^cH_2O that can be derived from these figures are different from those listed in the tables, where a correction for GFR was made.

[1] Kindly supplied by Dr. Robert Gaunt, Ciba Inc., Summit, N. J.

[2] 3-(3-Oxo-7α-acetylthio-17β-hydroxy-4-androskn-17α-yl)propionic acid-γ-lactone-(Aldactone).

[3] 3-Oxo-9α-fluoro-11β-dihydroxy-17α-pregn-4-ene-21-carboxylic acid (K salt). Both SC-9420 and SC-11929 were kindly supplied by Dr. Clarence L. Gantt, G. D. Searle & Co., Chicago, Ill.

TABLE I

The effects of varying salt intake on maximum urinary concentrating ability and T^cH_2O formation during mannitol diuresis

Dog	Preparation	Day	Serum Na	Serum K	GFR*	Maximum osmotic U/P ratio	Maximum observed $\frac{T^cH_2O}{GFR}\times100$	Derived $\frac{T^cH_2O}{GFR}\times100$	Slope	Calculated point of crossover†
			mEq/L	*mEq/L*	*ml/min*		*ml/100 ml GFR*	*ml/100 ml GFR*		*ml/min*
14	Na-fed		150	3.8	52	2.9	5.9	5.2	1.04	> 20
	Na-free	7	156	5.2	26	2.8	3.5	−0.8	0.77	4.3
29	Na-fed		157	3.4	70	3.3	5.0	4.2	0.91	> 20
	Na-free	7	152	4.1	54	2.5	1.7	−1.3	0.72	3.6
42	Na-fed		152	2.9	42	4.3	5.8	4.4	0.98	> 20
	Na-free	3	156	3.6	39	3.9	2.5	0.9	0.86	8.6
43	Na-fed		149	4.3	56	4.4	5.3	4.4	0.95	> 20
	Na-free	6	144	4.6	37	3.9	1.3	−0.2	0.74	5.4
47	Na-fed		149	4.5	81	4.0	1.6	1.4	1.02	> 20
	Na-free	7	152	5.3	66	2.8	0.8	−0.8	0.96	< 1.0
48	Na-fed		148	4.5	50	4.0	6.5	4.8	1.06	> 20
	Na-free	9	144	4.8	44	3.5	2.3	1.2	0.99	12.2
52	Na-fed		142	4.2	62	5.5	2.7	2.5	1.02	> 20
	Na-free	6	137	4.4	55	4.3	2.8	0.9	0.91	12.2
39	Na-fed		143	3.4	66	4.4	5.2	5.4	1.20	> 20
	Na-free	7	142	3.7	55	4.0	4.0	3.4	1.12	> 20
58	Na-fed		159	3.9	53		4.8	3.8	1.07	> 20
	Na-free	7	163	4.1	29		1.4	−0.1	0.94	6.7
62	Na-fed		149	3.8	64	4.1	1.7	1.5	0.94	> 20
	Na-free	7	143	4.7	60	3.2	0.5	0.6	0.97	16.7
66	Na-fed		142	4.5	73	4.0	3.8	3.7	0.99	> 20
	Na-free	6	143	4.7	54	4.0	3.1	1.6	0.75	11.6
67	Na-fed		143	4.5	45	4.5	4.3	4.1	0.95	> 20
	Na-free	6	141	4.0	41	3.6	1.6	1.3	0.89	9.1
Na-fed control										
Mean			149	4.0	60	4.1	4.4	4.1	1.01	> 20
SD			±1.8	±0.5	±12	±0.7	±1.6	±1.1	±0.08	
Na-free										
Mean			148	4.4	46	3.5	2.1	0.6	0.88	9.0
SD			±2.5	±0.5	±13	±0.6	±1.1	±1.2	±0.12	±4.6
Difference										
Mean			−0.8		−14	−0.6	−2.3	−3.5	−0.12	
p			> 0.500		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

* The glomerular filtration rate (GFR) represents the mean value for all collection periods. GFR remained relatively constant throughout all studies reported.

† This figure represents the intersection of the regression line with the isosmotic line. At this urine flow, the urine would become hypotonic.

thesia depress T^cH_2O formation. Nevertheless, to minimize possible effects of anesthesia on maximal urinary concentration, only bladder urine formed before the animals were anesthetized was used for this measurement.

A. Sodium-fed control. Sodium-fed controls under the conditions of water restriction and exogenous ADH had maximum osmotic U/P ratios of 4.4 ± 1.1. During mannitol diuresis T^cH_2O formation progressively increased until at moderate urine flows maximum values were attained. T^cH_2O then remained relatively constant as urine flow was further increased (11). At urine flows between 3 and 13 ml per minute, the regression of C_{Osm} on V was linear (Figure 1). In 30 control experiments the mean slope of the regression was 1.00 ± 0.08; the $T^c_MH_2O$ was 4.6 ± 1.5 ml per minute; and the $T^c_DH_2O$ was 3.9 ± 1.4 ml per minute. These figures are in agreement with some published control values in the dog (6, 12), but $T^c_MH_2O$ and maximum osmotic U/P ratios are lower than some others (13, 14). This is probably the result of a lower protein intake in our animals (15, 16).

At urine flows greater than 15 ml per minute, the slope decreased sharply. In no instance, however, did a sodium-fed dog elaborate hypotonic urine at a urine flow less than 20 ml per minute. This phenomenon has been pointed out by Raisz, Au and Scheer (17).

B. Sodium restricted. Maximum urinary concentration and T^cH_2O formation were determined in 12 dogs during periods of sodium feeding and then after salt restriction (Table I). Thus, each dog served as its own control. Salt restriction resulted in a mean fall in GFR of 23 per cent ($p < 0.001$) from control values. Maximum osmotic U/P ratios diminished slightly (from 4.1 to 3.5) but significantly ($p < 0.001$). $T^c_MH_2O$, in contrast, fell dramatically from 4.4 to 2.1 ml per minute ($p < 0.001$), while $T^c_DH_2O$ fell from a control value of 4.1 to 0.6 ml per minute. The slope diminished from 1.0 to 0.88 ($p < 0.001$), so that frequently at moderate urine flows a hypotonic urine resulted (Figure 2). There was no change in the serum concentration of either sodium or potassium.

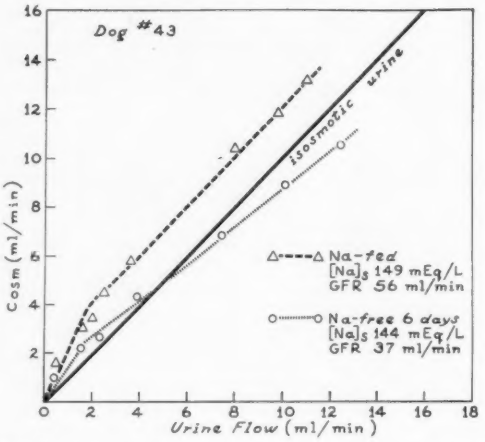

FIG. 2. THE EFFECT OF SODIUM DEPLETION ON T^cH_2O FORMATION DURING MANNITOL DIURESIS.

TABLE II

The effects of varying doses of exogenous aldosterone on maximum urinary concentrating ability and T^cH_2O formation during mannitol diuresis

Dog	Preparation	Dose	Serum Na	Serum K	GFR	Maximum osmotic U/P ratio	Maximum observed $\frac{T^cH_2O}{GFR} \times 100$	Derived $\frac{T^cH_2O}{GFR} \times 100$	Slope	Calculated point of crossover
		μg/kg body wt	*mEq/L*	*mEq/L*	*ml/min*		*ml/100 ml GFR*	*ml/100 ml GFR*		*ml/min*
39	Control		145	3.3	67	4.4	3.2	3.0	1.03	> 20
	Aldo.	30	150	3.9	62	3.5	3.9	3.2	0.93	> 20
	Aldo.	60	140	4.2	72	4.2	2.6	2.4	0.93	> 20
55	Control		146	4.2	40	5.3	4.0	2.2	1.00	> 20
	Aldo.	42	156	4.0	37	5.1	3.6	2.7	0.99	> 20
56	Control		146	3.9	55	4.3	3.1	2.3	1.02	> 20
	Aldo.	36	155	4.2	54	6.3	7.4	6.1	1.12	> 20
62	Control		149	3.8	64	4.1	1.7	1.5	0.94	> 20
	Aldo.	410	140	4.6	62	4.4	2.1	1.9	0.93	> 20
63	Control		147	4.0	39	5.6	3.6	4.0	0.96	> 20
	Aldo.	410	160	4.0	36	4.8	5.8	5.0	0.96	> 20
65	Control		144	4.5	43	3.2	3.8	3.3	0.95	> 20
	Aldo.	425	151	3.7	51	3.0	3.3	2.8	0.90	> 20
66	Control		142	4.5	73	4.0	3.8	3.7	0.99	> 20
	Aldo.	380	148	4.1	72	4.0	2.0	2.0	0.90	> 20
67	Control		143	4.5	45	4.5	4.3	4.1	0.95	> 20
	Aldo.	500	146	4.4	40	5.4	5.4	4.9	0.99	> 20
Control										
Mean			145	3.9	54	4.3	3.4	3.1	0.98	> 20
SD			±2	±0.5	±13	±0.7	±0.2	±0.8	±0.04	
Aldosterone										
Mean			149	4.2	53	4.4	3.8	3.2	0.95	> 20
SD			±7	±0.9	±14	±1.0	±1.9	±1.8	±0.7	
Difference										
Mean			+4	+0.2	−0.8	+0.1	+0.3	+0.1	−0.3	
p			> 0.05	> 0.20	> 0.50	> 0.50	> 0.50	> 0.50	> 0.20	

TABLE III

The effects of blocking endogenous aldosterone activity with spirolactones on T^cH_2O formation during mannitol diuresis in sodium-depleted dogs

Dog	Preparation, no. of days	Serum Na	Serum K	GFR	Maximum observed $\frac{T^cH_2O}{GFR}\times100$	Derived $\frac{T^cH_2O}{GFR}\times100$	Slope	Calculated point of crossover
		mEq/L	*mEq/L*	*ml/min*	*ml/100 ml GFR*	*ml/100 ml GFR*		*ml/min*
53	Na-free, 8	140	3.6	59	1.7	1.2	0.86	11.4
	Na-free, 15; + oral SC-9420, 7	133	3.8	45	3.5	2.5	0.88	15.8
62	Na-fed control	149	3.8	64	1.7	1.5	0.94	>20
	Na-free, 7	143	4.7	60	0.5	0.6	0.97	16.7
	Na-free, 14; + SC-11927, 10 mg, i.m., 7	138	4.7	59	2.0	0.3	0.64	7.8
66	Na-fed control	142	4.5	73	3.8	3.7	0.99	>20
	Na-free, 7	143	4.7	54	3.1	1.6	0.75	11.6
	Na-free, 14; + SC-11927, 10 mg, i.m., 7	143	4.9	52	1.5	−0.4	0.70	8.0
69	Na-fed control	145	4.1	47	4.6	3.7	0.99	>20
	Na-free, 10	146	4.0	42	3.3	2.0	0.74	11.2
	Na-free, 17; + SC-11927, 20 mg, i.m., 7	149	4.4	48	4.8	2.0	0.84	14.2
39	Na-fed control	147	3.8	72	5.8	5.0	1.12	>20
	Na-free, 10	143	4.4	54	4.5	0.3	0.81	12.3
	Na-free, 16; + SC-11927, 20 mg, i.m., 6	140	4.7	44	2.6	0.8	0.87	13.9
71	Na-fed control	146	3.7	64	5.6	4.8	1.07	>20
	Na-free, 8	145	4.5	44	2.0	0.1	0.76	6.9
	Na-free, 14; + SC-11927, 100 mg, i.m., 6	136	5.0	45		0.8	0.89	7.3

C. Aldosterone and aldosterone antagonists. Sodium restriction is known to cause a rise in endogenous aldosterone secretion. If aldosterone caused a significant degree of sodium reabsorption in the proximal tubule, it might, by making less sodium available for reabsorption in the ascending limb of the loop of Henle, be responsible for the impairment in urinary concentration observed in sodium deprivation.

To examine this hypothesis, ten dogs were maintained on the sodium-containing diet. Studies were performed after 3 to 5 days and were then repeated 4 to 7 days later. Two hours before the repeat study, 30 to 500 μg of DL-aldosterone per kg body weight was given i.v. The results are summarized in Table II. No significant changes in GFR, slope, $T^c_MH_2O$, maximum osmotic U/P ratio, serum sodium and serum potassium were observed. One typical study is shown in Figure 3. The activity of the aldosterone preparation was attested to by a decreased sodium excretion rate and an increased potassium excretion rate at any given urine flow when compared with the control study in the same dog. It appeared then that exogenous aldosterone alone was incapable of diminishing T^cH_2O formation.

To explore the possibility that excessive endogenous aldosterone, when coupled with diminished filtered sodium, might still be an essential component in depressing T^cH_2O, further studies were done. Five sodium-restricted dogs displaying typical defective urinary concentrating ability were continued on sodium deprivation and given an aldosterone antagonist for 7 days. The results (Table IIII) show that by blocking the action of aldosterone, neither T^cH_2O formation, maximum urinary concentrating ability, nor GFR was restored to normal (Figure 4).

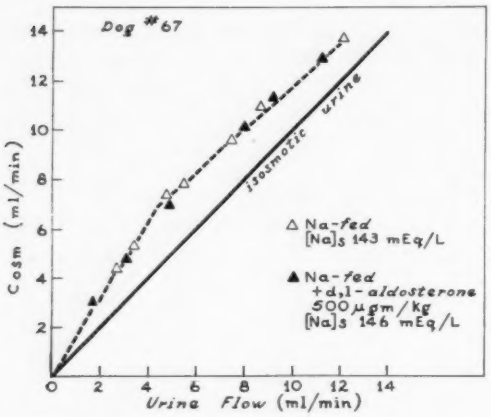

FIG. 3. THE EFFECT OF EXOGENOUS ALDOSTERONE ON T^cH_2O FORMATION DURING MANNITOL DIURESIS IN A SODIUM-FED DOG.

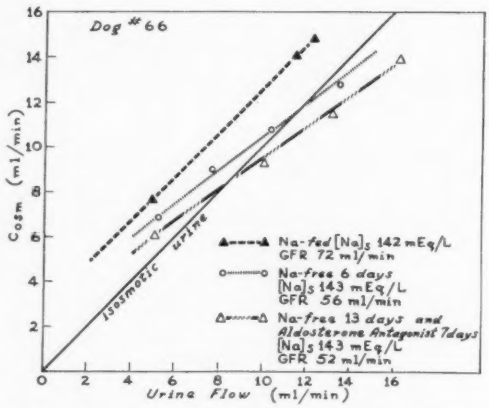

FIG. 4. THE EFFECT OF BLOCKING ENDOGENOUS ALDOSTERONE ACTIVITY WITH SC-11927 ON T^cH_2O FORMATION DURING MANNITOL DIURESIS IN A SODIUM-DEPLETED DOG.

It seems that the defect in renal water conservation produced by sodium restriction is not due to increased endogenous aldosterone secretion.

D. Raising GFR during sodium restriction. Inasmuch as the defect in urinary concentration was displayed only when GFR had fallen below control levels, the effect of increasing GFR in the

TABLE IV

The effects of increasing GFR with methylprednisolone on T^cH_2O formation during mannitol diuresis in sodium-depleted dogs

Dog	Preparation, no. of days	Serum Na	Serum K	GFR	Maximum observed $\frac{T^cH_2O}{GFR}\times 100$	Derived $\frac{T^cH_2O}{GFR}\times 100$	Slope	Calculated point of crossover
		mEq/L	*mEq/L*	*ml/min*	*ml/100 ml GFR*	*ml/100 ml GFR*		*ml/min*
43	Na-fed control	149	4.3	56	5.3	4.4	0.95	>20
	Na-free, 3	144	4.6	37	1.3	−0.2	0.74	5.4
	Na-free, 7; + acute methylprednisolone	145	3.9	54	3.3	1.9	0.84	12.5
47	Na-fed control	149	4.5	81	1.6	1.4	1.02	>20
	Na-free, 7	152	5.3	66	0.8	−0.8	0.96	< 1.0
	Na-free, 7; + methylprednisolone, 6	145	3.5	105	2.5	1.7	0.84	>20
48	Na-fed control	148	4.5	50	6.5	4.8	1.06	>20
	Na-free, 9	144	4.8	44	2.3	1.2	0.99	12.2
	Na-free, 7; + methylprednisolone, 6	140	4.1	59	5.6	4.7	1.10	>20
67	Na-fed control	143	4.5	45	4.3	4.1	0.95	>20
	Na-free, 6	141	4.0	41	1.6	1.3	0.89	9.1
	Na-free, 9; + methylprednisolone, 4	135	4.1	50	4.0	4.0	1.03	>20
62	Na-fed control	149	3.8	64	1.7	1.5	0.94	>20
	Na-free, 14	138	4.7	59	2.0	0.3	0.64	7.8
	Na-free, 16; + methylprednisolone, 4	150	4.3	64	6.2	5.5	1.04	>20

face of sodium restriction was examined. Five dogs, displaying the usual defect on sodium restriction, were continued on the sodium-free diet and given methylprednisolone. In all five there was an elevation of GFR to or above control levels (Table IV). In four experiments $T^c_MH_2O$ reached values at or close to those of their controls, but in one the restitution was only to about half normal. In this animal (Dog 43), the methylprednisolone was given i.v. only 20 minutes prior to study, whereas in those with complete correction it had been given for several days. One study is shown in Figure 5.

Methylprednisolone was chosen as the agent to increase filtration rate because it has negligible mineralocorticoid activity. Although it has been postulated that glucocorticoid inhibits back diffusion of water in the renal tubule (18), this effect would serve only to enhance the defect rather than correct it. Attempts to raise GFR in the salt-deprived dogs with dextran, aminophylline, and glycine infusions were unsuccessful.

E. Hyponatremia. The defect in urinary concentration during sodium deprivation appears to be intimately related to a reduction in GFR. Whether or not this is simply the consequence of a diminished filtered load of sodium was explored

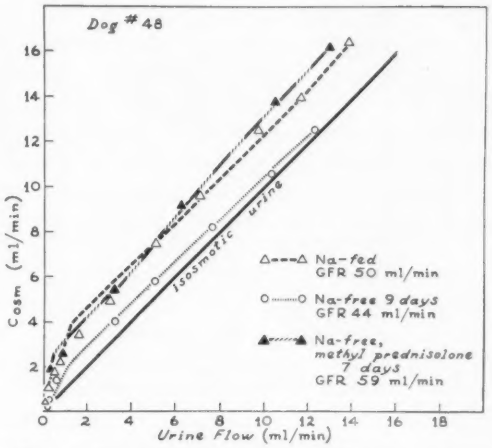
Dog # 48
Csm (ml/min)
Urine Flow (ml/min)
Isosmotic urine
Δ---Δ Na-fed GFR 50 ml/min
O·····O Na-free 9 days GFR 44 ml/min
▲-·-▲ Na-free, methyl prednisolone 7 days GFR 59 ml/min

FIG. 5. THE EFFECT OF RAISING GFR WITH METHYLPREDNISOLONE ON T^cH_2O FORMATION DURING MANNITOL DIURESIS IN A SODIUM-DEPLETED DOG.

TABLE V

The effects of hyponatremia on T^cH_2O formation during mannitol diuresis in sodium-fed and sodium-depleted dogs

Dog	Preparation, no. of days	Serum Na	Serum K	GFR	Maximum osmotic U/P ratio	Maximum observed $\frac{T^cH_2O}{GFR}\times100$	Derived $\frac{T^cH_2O}{GFR}\times100$	Slope	Calculated point of crossover
		mEq/L	*mEq/L*	*ml/min*		*ml/100 ml GFR*	*ml/100 ml GFR*		*ml/min*
12	Na-free, 8	152	4.4	38	3.0	1.9	−1.5	0.81	4.2
	Na-free, 11; + vasopressin	149	4.3	46	3.3	2.7	−0.3	0.74	6.5
	Na-free, 14; + vasopressin + H_2O	120	4.8	42	1.7	2.0	−1.1	0.80	5.0
23	Na-fed control	149	3.1	40	2.7	5.2	3.2	0.94	>20
	Na-fed + vasopressin	145	4.0	42	3.4	8.4	6.2	1.10	>20
	Na-fed + vasopressin + H_2O	126	4.1	41	2.3	5.2	4.5	1.02	>20
58	Na-fed control	159	3.9	53	5.5	4.8	3.8	1.07	>20
	Na-fed + vasopressin + H_2O	126	4.2	48	4.6	3.6	2.9	0.95	>20
68	Na-fed control	149	3.9	59	3.8	6.8	5.9	1.05	>20
	Na-fed + vasopressin + H_2O	128	3.4	63	5.6	6.3	5.3	1.06	>20
	Na-free, 4; + vasopressin	117	3.6	56	4.8	5.1	4.9	0.73	16.4
69	Na-fed control	145	4.1	47	2.5	4.6	3.7	0.99	>20
	Na-fed + vasopressin + H_2O	121	3.5	47	3.4	4.9	3.8	0.98	>20
	Na-free, 10	146	4.0	42	3.0	3.3	2.0	0.74	11.2
	Na-free, 4; + vasopressin	120	3.8	41	2.1	2.8	−1.4	0.59	6.6

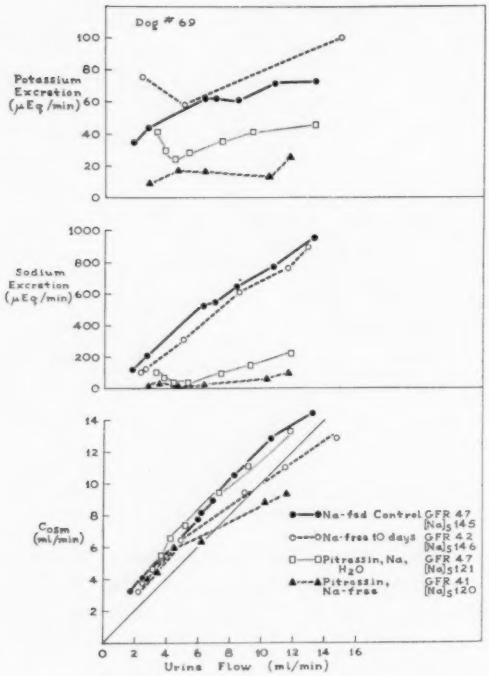
Dog # 69
Potassium Excretion (μEq/min)
Sodium Excretion (μEq/min)
Cosm (ml/min)
Urine Flow (ml/min)
Na-fed Control GFR 47 [Na]S 145
Na-free 10 days GFR 42 [Na]S 146
Pitressin, Na, H2O GFR 47 [Na]S 121
Pitressin, Na-free GFR 41 [Na]S 120

FIG. 6. VARIATIONS IN T^cH_2O FORMATION, SODIUM EXCRETION, AND POTASSIUM EXCRETION DURING MANNITOL DIURESIS IN A DOG SODIUM-FED AND THEN SODIUM-DEPRIVED WITH NORMAL AND LOW LEVELS OF SERUM SODIUM.

in another group of studies. In these studies filtered sodium was diminished by inducing hyponatremia with chronic vasopressin administration and excess water. Filtered sodium was reduced 13, 28, 8, and 17 per cent (average, 17 per cent) in these four hyponatremic dogs (Table V) compared with an average 23 per cent reduction in filtered sodium in the salt-restricted dogs. Despite comparable reduction of filtered sodium during hyponatremia there was no change in T^cH_2O formation. In Dog 69 (Figure 6) a 17 per cent reduction in filtered sodium during hyponatremia had no effect on T^cH_2O formation, whereas a 10 per cent reduction in filtered sodium during salt deprivation drastically reduced T^cH_2O formation. Hence it is concluded that diminished filtered sodium per se does not reproduce the defect in T^cH_2O formation observed in salt-restricted dogs.

DISCUSSION

Sodium restriction in dogs impairs urinary concentration in an unusual fashion: the ability to produce maximally concentrated urine is only slightly impaired, whereas the formation of T^cH_2O is curtailed to the extent that hypotonic urine is excreted at moderate urine flows. Thus salt deprivation profoundly alters the normal function of the renal concentrating mechanism.

According to current concepts of urine concentration, distal tubular fluid equilibrates to isotonicity under the influence of antidiuretic hormone before entering the collecting tubule, even during severe mannitol diuresis (3). In the collecting tubule water is abstracted along osmotic gradients into the hypertonic medullary interstitium, resulting in T^cH_2O formation and the elaboration of a hypertonic urine. Any factor, therefore, that interferes with medullary hypertonicity will alter both the ability to produce maximally concentrated urine and T^cH_2O formation. However, a reduction in medullary hypertonicity alone will not result in the formation of hypotonic urine. Since there is no convincing evidence that the collecting tubule is capable of reabsorbing solute in excess of water during either diuresis or antidiuresis, the excretion of a hypotonic urine is *prima facie* evidence that the fluid entering the collecting tubule is hypotonic.[4]

A fall in medullary hypertonicity, however, could result in excretion of hypotonic urine if, in the dog (in contrast to the rat), fluid issuing from the distal tubule were always hypotonic. In the presence of ADH activity, the delivery of hypotonic urine to the collecting tubule would normally be obscured by extraction of large amounts of T^cH_2O. If this were the case, impairment of medullary hypertonicity during salt deprivation could in fact result in hypotonic urine in mannitol diuresis. Although most investigators have assumed that in the presence of ADH the fluid leaving the distal tubule is always isotonic in man, dog and rat, only in the rat is direct micropuncture evidence available to establish this point (3). The fact that man and dog excrete hypotonic urine in the presence of maximum antidiuretic hormone in certain experimental and pathological condi-

[4] Wirz (19) has published observations on six water diureses: the osmotic pressure of ureteral urine was higher than that of the distal tubule in one instance, the same in two instances, and slightly lower in three. Indeed, Gottschalk and Mylle (3) have shown that the osmotic pressure of ureteral urine is higher than that of the distal tubule in diabetes insipidus.

tions (4, 6, 9, 13, 16, 20–22) while the rat, in these same conditions, shows only decreased urinary concentration but never hypotonic urine, suggests that the equilibration of fluid in the distal tubule may vary between these species.

If, then, in the dog hypotonic fluid were always entering the collecting tubule the concentrating defect of sodium restriction could be entirely explained by a decrease in medullary hypertonicity. A possible mechanism by which sodium deprivation might reduce medullary hypertonicity is a reduced delivery of sodium to the loop of Henle, as a result of either increased proximal tubular reabsorption due to enhanced aldosterone secretion or diminished sodium loads as a result of a fall in GFR. The failure of aldosterone in normal dogs to produce, and spirolactone in sodium-restricted dogs to correct the defect is strong evidence that augmented aldosterone secretion is not responsible for the impaired urinary concentrating ability. The only factor in these studies that was consistently related to the defect was a chronic reduction in GFR. The fact that administration of methylprednisolone to salt-restricted dogs both raised GFR and restored the capacity to form T^cH_2O suggests that a reduction in GFR is in some manner implicated in the observed defect in urinary concentration.

A reduction in GFR, by diminishing the amount of filtered sodium, might result in impaired urinary concentration by reducing the delivery of sodium to the loop of Henle. In the hyponatremic dogs, however, the reduction in filtered sodium, comparable in degree with that produced by sodium restriction, failed to impair concentrating ability. It therefore seems unlikely that the amount of filtered sodium is the determining factor.

It is conceivable, however, that when filtered sodium is reduced via a lowered GFR, less sodium reaches the loop of Henle than when a similar reduction in filtered sodium is induced by hyponatremia. This possibility was examined by comparing the excretion of sodium and potassium during mannitol diuresis in hyponatremic and salt-restricted dogs (Figure 6). In salt-deprived dogs with normal serum sodium the same amount of sodium was excreted at any given urine flow as in sodium-fed dogs, suggesting that during salt deprivation normal amounts of sodium reach the loop of Henle. In contrast, sodium and potassium excretion are much less at comparable urine flows in hyponatremic dogs. The reduction in potassium excretion in these dog suggests that less sodium traverses the loop of Henle to reach the distal tubule. Therefore, despite the fact that normal amounts of sodium pass through the loop in the sodium-deprived dogs, T^cH_2O formation is defective. On the other hand, reduced amounts of sodium leave the loop of Henle in both the sodium-fed and sodium-depleted hyponatremic dog; however, T^cH_2O is markedly impaired in hyponatremic dogs deprived of sodium but normal in those that are sodium-fed. Therefore the reduction in GFR, that appears to be implicated in defective T^cH_2O formation in sodium deprivation, does not appear to mediate this defect by reducing the delivery of sodium to the loop of Henle. It is not clear, therefore, how a reduced GFR could result in a decrease in medullary hypertonicity. Moreover, the observation that the maximum osmotic U/P ratio is only slightly altered by sodium restriction suggests that reduction in medullary hypertonicity is not the mechanism involved.

If, on the other hand, the dog is like the rat in that distal tubular fluid normally equilibrates to isotonicity before entering the collecting tubule, no degree of impairment of medullary hypertonicity could explain the excretion of hypotonic urine, and another mechanism must be involved. An alternative explanation is that sodium deprivation in some ways impairs the permeability of the distal tubule to water and thus prevents equilibration of distal tubular fluid to isotonicity. Under these circumstances mannitol diuresis would sweep increasing quantities of hypotonic fluid into the collecting tubule, thus obscuring T^cH_2O formation and resulting in excretion of hypotonic urine unrelated to either sodium transport by the loop of Henle or medullary tonicity. The manner in which sodium depletion could alter the permeability of the distal tubule in this fashion is not clear.

SUMMARY

Urinary concentrating ability was studied in dogs before and after sodium restriction by measuring maximum urinary concentration and T^cH_2O formation during mannitol diuresis. Sodium deprivation resulted in a slight fall in maximum osmotic U/P ratios and a marked fall in T^cH_2O

formation so that hypotonic urine was excreted at moderate urine flows. This alteration in urinary concentrating ability was not due to an increased aldosterone secretion, since exogenous aldosterone failed to produce it in sodium-fed dogs and aldosterone antagonists failed to correct it in sodium-deprived dogs. The defect, however, was always associated with a fall in glomerular filtration rate (GFR) and was corrected by raising GFR in salt-restricted dogs with methylprednisolone. That diminished GFR did not produce the defect by decreasing the amount of sodium reaching the loop of Henle was shown by the ability of sodium-fed hyponatremic dogs to form normal T^cH_2O despite a reduction in sodium delivery to the loop of Henle to levels far below those of normonatremic sodium-deprived dogs. It is concluded that the defect in urinary concentration seen in sodium deprivation is due to the failure of distal tubular fluid to equilibrate to isotonicity before reaching the collecting tubule.

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CLINICAL AND PHYSIOLOGICAL OBSERVATIONS IN A PATIENT WITH AN IDIOPATHIC DECREASE IN THE THYROXINE-BINDING GLOBULIN OF PLASMA *

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Since its discovery in 1952 (1), the thyroxine-binding globulin of plasma (TBG) has been the subject of extensive investigation designed to elucidate its physicochemical characteristics, alterations in abnormal states, and physiological function. Recent reviews have summarized available knowledge in this area (2, 3).

The present report describes a male patient whose plasma appears to be virtually devoid of thyroxine binding by TBG. This unusual abnormality, fortuitously discovered, afforded a unique opportunity to assess further the role of TBG in regulating the peripheral metabolism of the thyroid hormone. A number of physiological studies was therefore performed in this patient, and the results are reported herein. While these studies were in progress, clinical features of a male patient with a similar thyroxine-binding abnormality were reported by Tanaka and Starr (4). It is likely that the physiological disturbances currently described were also present in the earlier case and that both patients are affected by the same distinct, though rare, disorder in plasma protein metabolism.

CASE HISTORY

History. F.B., a 58 year old unmarried white male, was admitted to the Harvard Medical Service of the Boston City Hospital on September 14, 1958. His chief complaint at that time was increasingly severe exertional dyspnea, orthopnea, and episodes of paroxysmal nocturnal dyspnea of 2 years' duration. He had taken digitalis irregularly during that period, but had had none during the year prior to entry. The patient had been known to have a heart murmur since childhood. Symptoms of acute rheumatic fever were denied. The patient's only other complaint was weakness of the arms and legs of 2 year's duration. This was especially prominent in the distal musculature and was of moderate, but not incapacitating, severity.

Details of early development were not readily recalled, but pubertal maturation apparently began at about age 14. At age 16 a bilateral inguinal herniorrhaphy was performed. Subsequently, if not immediately thereafter, normal masculine psychosexual development ceased. Libido was minimal throughout the patient's life, although rare erections and nocturnal emissions occurred. Pubic and axillary hair remained scant, and balding did not take place. The patient shaved infrequently.

There were no other symptoms referable to the endocrine organs, including the thyroid, adrenals and pituitary gland, and no symptoms of an intracranial lesion. Review of systems was otherwise negative.

The patient's parents had died when he was a young boy; he had no siblings. He knew of no other close relatives who were alive. The patient was raised by members of a religious order and was living and serving as janitor in their institution at the time he entered the hospital.

Physical examination. T, 98.8°; P, 100; R, 20; BP, 130/70. Salient features of the physical examination were confined to the cardiovascular, endocrine and neuromuscular systems. Examination of the heart revealed cardiomegaly and a prominent precordial heave. Cardiac rhythm was regular. The pulmonic second sound was increased, split, and louder than the aortic second sound. The first sound in the mitral area was booming; there was no opening snap. A grade 4, harsh systolic murmur, radiating widely, was audible over the entire precordium, but was heard best in the fourth left intercostal space in the parasternal area.

Bilateral herniorrhaphy scars and reducible herniae were present. Small, firm testes were felt in the scrotal sac. The phallus was normal in size, the prostate small and firm. Pubic hair was scant, the escutcheon female. Only a few fine strands of axillary hair were present. The skin was fine and smooth, typical of that associated with hypogonadism. Skeletal proportions were not eu-

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nuchoid. There was a slight temporal calvietes, but no other balding.

There were no peripheral stigmata of hyper- or hypothyroidism. There were no pigmentary abnormalities. The eyes were highly myopic, but there were no cataracts. Visual fields were normal to gross confrontation.

There was moderate weakness of the distal musculature of the arms and legs with atrophy of the interosseous muscles. No myotonia was evident. Deep tendon reflexes and sensory examination were normal.

Laboratory. The urine and blood were within normal limits. Lumbar puncture was normal. Serum chemistries included: urea nitrogen, 15 mg per 100 ml; glucose (fasting), 100 mg per 100 ml; calcium, 10.2 mg per 100 ml; phosphorus, 3.1 mg per 100 ml; cholesterol, 210 mg per 100 ml; sodium, 138 mEq per L; potassium, 4.1 mEq per L; chloride, 104 mEq per L; CO_2, 25.6 mEq per L. Serum iron concentration was 110 μg per 100 ml and iron-binding capacity 320 μg per 100 ml, both within normal limits.

Skull films were normal. A bone survey revealed no abnormalities; the epiphyses of the iliac crests were closed. Visual fields were within normal limits. Twenty-four hour urinary excretion of 17-ketosteroids was 9.9 mg (5), of 17-hydroxycorticoids, 4.3 mg (6).[1] Urinary gonadotropins were positive at 50 and equivocal at 100 mouse units per 24 hours (7).[2]

Twenty-four hour thyroidal uptake of I^{131} was 28 per cent. BMR's ranged between -5 and $+4$ per cent. Serum protein-bound iodine (PBI) was 2.0 μg per 100 ml (8).

Course. The patient's cardiac complaints responded well to digitalization and a low salt diet. Mercurial diuretics were not employed. Cardiac catheterization studies were interpreted as consistent with a persistent left vena cava and atrial septal defect, probably of the *secundum* type.

In view of the patient's obvious hypogonadism, a thorough endocrine evaluation was undertaken. The associated subnormal PBI, repeatedly confirmed, strongly raised the possibility of pituitary hypofunction, but this diagnosis seemed clearly excluded by the neurological and X-ray examinations and by other endocrinological findings, especially the increased urinary excretion of gonadotropins. The dissociation between the PBI and other laboratory, as well as clinical, indices of thyroid function raised the possibility of abnormal binding of thyroxine in the patient's plasma. This hypothesis was confirmed by repeated electrophoretic studies of the patient's serum, all of which revealed a virtual absence of TBG.[3]

Studies of the peripheral turnover of I^{131}-labeled thyroxine were then performed. Several days after these were complete, the left inguinal hernia became incarcerated and emergency repair was performed. At this time the testis was seen to be grossly atrophic and a testicular biopsy was taken. In view of the muscular weakness, a biopsy of the anterior tibial muscle and overlying skin was also made. The latter revealed no abnormality. Histologically, the testis was fibrotic, with atrophy and fibrous replacement of tubular elements and decrease in interstitial cells, consistent with chronic restriction of blood supply. Genetic sex was masculine, as assessed in both testicular and cutaneous biopsies.

When the patient's convalescence was complete, oral administration of diethylstilbestrol, 30 mg daily, was begun. This was continued for 7 weeks. During the latter portion of this period, the patient developed increasing dyspnea, pretibial edema, marked areolar pigmentation, and pronounced tender gynecomastia. Urinary excretion of gonadotropin declined to less than 5 mouse U per 24 hours. Electrophoretic studies of hormonal binding in the patient's serum were performed at weekly intervals during the administration of diethylstilbestrol. During the last 10 days of this regimen, studies of the peripheral metabolism of labeled thyroxine were repeated.

Two months later, after clinical evidence of estrogenic effect had disappeared, a course of adrenocortical suppressive therapy was begun. The patient was given 10.0 mg prednisolone[4] by mouth, each day for a period of 6 weeks. Under this treatment urinary 17-ketosteroids declined to 1.1 mg daily. Bloods were drawn weekly for electrophoretic analysis of thyroxine binding.

After gradual withdrawal of corticoid therapy, a 6-week course of sodium-L-thyroxine, 0.2 mg by mouth daily, was instituted. The patient experienced no symptoms from this medication; physical examination, PBI, and electrophoretic findings with regard to thyroxine binding remained unaltered. Twenty-four hour thyroidal uptake of I^{131} was suppressed from its control value of 28 per cent to 6 per cent, however.

After withdrawal of exogenous thyroid hormone, the patient remained well and has continued thus, save for one episode of congestive heart failure that followed discontinuance of digitalis, which quickly responded to redigitalization. Bloods have subsequently been obtained at intervals for electrophoretic analysis; these have continued to show a virtual absence of TBG.

MATERIALS AND METHODS

Thyroxine-binding capacities of individual serum proteins were determined by zonal electrophoresis in filter paper of serum enriched with variable quantities of I^{131}-labeled and stable L-thyroxine.

[1] Performed at the Boston Medical Laboratory, Boston, Mass. Normal values: 17-ketosteroids, 10 to 24 mg per 24 hours; 17-hydroxycorticoids, 1 to 10 mg per 24 hours.

[2] Performed at the Gynecological-Endocrinological Laboratory, Peter Bent Brigham Hospital, Boston, Mass.

[3] In the present report, for purposes of brevity, a change in the thyroxine-binding activity of TBG will be referred to as a change in TBG; no implications concerning the absolute concentration of the protein are intended.

[4] 11β,17α,21-trihydroxy-4-pregnadiene-3,20-dione.

I^{131}-labeled hormones (L-thyroxine, T_4; 3,5,3′-L-triiodothyronine, T_3; 3,5,3′-triiodothyroacetic acid, triac; 3,5,3′5′-tetraiodothyroacetic acid, tetrac) obtained from a commercial source,[5] were 90 to 95 per cent pure, as assessed chromatographically, and were diluted with 1.0 per cent human serum albumin to a final concentration of 160 μc per ml immediately upon arrival. This procedure reduced both the degradation of labeled compounds and their adsorption to glassware. Nevertheless, labeled materials were not employed for studies after 1 week of storage in this form.

The procedure employed in preparing samples of serum for the electrophoretic estimation of thyroxine binding by TBG and thyroxine-binding prealbumin (TBPA) was as follows:

To 6.2 ml of serum, 75 μl of I^{131}-labeled T_4 in human serum albumin was added, yielding a final concentration of approximately 2 μc per ml (Solution I). A 3.0-ml aliquot of Solution I was pipetted into a separate tube, and to this was added 100 μl of solution of 1.0 per cent human serum albumin containing 14.0 μg of stable T_4. This yielded a solution containing 451 μg of added stable T_4 per 100 ml (Solution G). Aliquots of Solution I, Solution G, and mixtures of the two were employed to prepare samples containing 87, 112.5, 130, 340, 395, and 451 μg of added stable T_4 per 100 ml. The three lower concentrations were employed to calculate the binding capacity of TBG and the three higher concentrations, the binding capacity of TBPA.[6] Two additional samples of serum containing I^{131}-labeled but no stable T_4 were prepared. The first, Solution A, is a mixture of equal parts of Solution I with fresh serum (1 μc I^{131} per ml) and the second (Solution H) a 1:10 dilution of Solution A with fresh serum (0.1 μc I^{131} per ml). In general, the specific activity of the I^{131}-labeled T_4 is such that these two samples contain approximately 4 μg and 0.4 μg of added stable T_4 per 100 ml, respectively. Electrophoresis was performed in sheets of Whatman no. 3 filter paper, 22.5 × 12 inches, large enough to accommodate the 8 separate specimens. After being moistened in buffer medium and blotted, filter paper sheets were placed upon a carefully ground, siliconized glass plate,[7] 15 inches long, 13 inches wide, and 1 inch thick. Aliquots of serum (20 μl) were then applied in thin lines, equidistant from the ends of the sheet, to areas carefully predetermined from a template. Usually, duplicate aliquots of serum were applied to a second filter-paper sheet carefully placed directly upon the first sheet. A minute quantity of bromphenol blue (BPB) was applied to each serum band to serve as a marker for the electrophoretic migration of albumin. Sheets were then covered by a second glass plate, similar in dimension to the first. When, as is often the case, it is desired to analyze an additional 8 samples upon the same machine, a thin glass plate (13 × 15 × ⅛ inch) is placed upon the first pair of duplicate sheets. A second pair of sheets containing the additional samples can then be placed upon the thin plate and the entire "sandwich" covered by the second thick glass plate. To minimize evaporation, the hiatus between the top and bottom glass plates at the front and rear of the apparatus was sealed with cellophane tape. At the lateral margins of the plates, filter paper sheets largely filled this hiatus and were allowed to hang into buffer baths. Gravitational flow of buffer across the filter paper was eliminated by means of a horizontal leveling device in the buffer reservoirs.

Buffer systems employed in the present studies were either 0.05 M veronal or a (hydroxymethyl) aminomethane (Tris)-maleate buffer, both at pH 8.6.[8] Through platinum electrodes immersed in the buffer reservoirs, a potential of 110 was applied across the filter paper for a period sufficient to produce approximately a 4-inch migration of the serum albumin. This usually required about 18 hours at normal room temperature. No attempts were made to cool the apparatus, as heating is only slight under these conditions.

When migration of proteins was sufficient, filter paper sheets were dried. One sheet was set aside for radioautography and subsequent staining with BPB, making possible the localization of major protein components. The second sheet was cut into 8 strips and, except in the case of Sample H, radioactive scanning of individual electrophoretograms was performed by means of a continuous scanner and dual-channel recorder. Areas under the individual peaks were determined with an electronic integrator whose output is recorded synchronously with the scan itself. The distribution of labeled T_4 was calculated from the recorded integral as the proportion of added hormone bound by each of the demonstrated binding proteins. The total quantity of labeled T_4 bound by each protein per unit volume of serum was calculated as the product of the proportion bound and the estimated total concentration of T_4 in the specimen. The latter estimate is derived from the total concentration of added T_4 (I^{131}-labeled plus stable) plus an estimate of endogenous T_4, assuming the endogenous PBI to be comprised entirely of this hormone. Sample H was divided into segments 0.5 cm wide; these were counted individually in a well-type scintillation counter.

In view of the decreased thyroxine-binding capacity of TBG in the present patient's serum, samples were also assayed which had been enriched with both labeled and stable T_4 in a number of concentrations below the usual lowest enrichment with 87 μg of stable T_4 per 100 ml.

The concentration of the major electrophoretically distinguishable groups of plasma proteins was assessed by densitometric scanning of electrophoretic strips stained with BPB.

[5] Abbott Laboratories, Oak Ridge, Tenn.

[6] Evidence that thyroxine-binding sites of TBG and TBPA in normal serum are saturated at the concentrations indicated has been presented briefly in a previous report (*2*) and will be presented in detail in a later communication.

[7] Available from the Pittsburgh Plate Glass Co., Boston, Mass.

[8] Preparation of the Tris-maleate buffer has been described in an earlier communication (*2*). The final solution is 0.073 M with respect to both Tris and maleate.

Thyroxine turnover studies. Analyses of the peripheral turnover of I^{131}-labeled T_4 *in vivo* were performed according to methods described in detail elsewhere (9). Serial samples of serum and complete 24-hour urinary collections were obtained daily for 8 to 10 days after the intravenous administration of a tracer dose of I^{131}-labeled T_4. These were analyzed for their concentration of I^{131}. Aliquots of urine were also subjected to a butanol extraction procedure, similar to that described for serum (10), and the proportion of total urinary I^{131} present as butanol-extractable I^{131} was determined. The several functions calculated and their derivations are described in the Appendix.

Red blood cell uptakes of I^{131}-labeled hormones. Blood was drawn from a single normal donor (Type O) into a heparinized syringe. After centrifugation, plasma was removed and the erythrocytes were washed 4 times in 3 volumes of normal saline. After the final wash, sufficient normal saline was added to bring the red blood cell suspension to a standard hematocrit. A sufficient volume of the suspension was pipetted into a series of 13 × 100 mm counting tubes to provide 0.5 ml of packed red blood cells in each. Samples of fresh plasma (oxalate anticoagulant) from the patient and from a group of normal control subjects were enriched with 0.02 μc per ml of I^{131}-labeled compounds; 0.5-ml aliquots of plasma were pipetted into tubes containing the packed red blood cells. Tubes were stoppered and agitated at 37° C for 1 hour. The radioactivity in each tube was then assessed directly in a well-type scintillation counter. Tubes were centrifuged, the plasma removed by suction and the red blood cells were then washed 5 times with 3 volumes of physiological saline solution at room temperature. After the last wash, 0.5 ml of saline was added to the packed red blood cells to bring the final volume to 1 ml. The cells were resuspended, and the radioactivity in the tube was again measured.

Red blood cell uptakes of labeled hormones were calculated as the ratio of final to initial counting rates, expressed as a per cent. All analyses were performed in duplicate. Electrophoretic analyses of thyroxine-protein-binding interactions in the sera studied were performed in all instances.

RESULTS

Electrophoretic analyses (Figures 1, 2; Table I). Repeated electrophoretic analyses of the patient's serum failed to reveal more than a trace of T_4 in the inter-α-globulin (TBG) area, during electrophoresis of serum in either the Tris-maleate or the veronal buffer systems.[9] Radioactivity in the TBG zone was most frequently found when electrophoretograms of serum enriched with only 0.4 μg of T_4 per 100 ml were cut into strips and counted in a well counter. Even at this concentration, however, no more than an approximate 5 per cent of total radioactivity was localized in the TBG zone. Often, both before and during administration of diethylstilbestrol, no TBG could be demonstrated. In sera enriched with approximately 4.0 μg of T_4 per 100 ml, a small upward convexity in the radioactive scan was occasionally seen in the TBG zone. This was evident on radioautographs as a faint zone of darkening. Direct well counting of such strips indicated that no more than 2 to 3 per cent of added radioactivity was localized with TBG. In sera enriched with higher concentrations of T_4, no band in the TBG zone could be discovered. It thus appears likely that this patient's serum was either not completely or not always devoid of TBG. The binding capacity of TBG, when demonstrable, could not be assessed with any accuracy but clearly seemed no more than a small fraction of 1 μg of T_4 per 100 ml of serum.

Thyroxine-binding by TBPA was evident in all specimens subjected to electrophoresis in the Tris-maleate buffer system. The thyroxine-binding capacity of TBPA was usually near the normal mean of approximately 120 μg of T_4 per 100 ml of serum

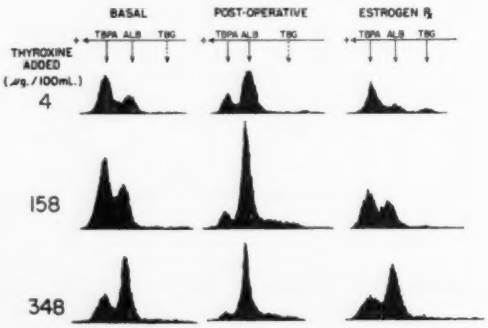

FIG. 1. THE EFFECT OF INGUINAL HERNIORRHAPHY AND OF DIETHYLSTILBESTROL ON THE BINDING OF THYROXINE IN THE SERUM OF A PATIENT WITH DECREASED THYROXINE-BINDING GLOBULIN (TBG). Postoperative serum obtained 2 days after surgery. Serum obtained during therapy (obtained after 5 weeks of treatment with diethylstilbestrol, 30 mg daily, p.o.). Curves shown are scans of I^{131}-labeled thyroxine added in the indicated concentrations to sera subjected to electrophoresis in Tris-maleate buffer, pH 8.6. The small peak seen in the TBG zone during estrogenic therapy was not evident in other samples obtained during the same treatment period.

[9] Values described for thyroxine-binding by TBG and TBPA represent the means of those found during electrophoretic analysis of at least 3 specimens obtained during each treatment period.

(2), but varied with the patient's clinical state, declining to subnormal values during exacerbations of congestive heart failure or in the period following emergency inguinal herniorrhaphy. A zone of radioactivity corresponding in electrophoretic migration to that of serum albumin was the only other area in which added I^{131}-labeled T_4 was localized.

As previously reported (11, 12), I^{131}-labeled triac and tetrac were bound primarily to TBPA and secondarily to albumin during electrophoresis in either Tris-maleate or veronal buffers. At standard concentrations of added labeled compounds, the percentile distribution of the derivatives between these two proteins in the patient's serum, obtained during the control state, was not appreciably different from normal.

During the administration of estrogen, prednisolone, or sodium-L-thyroxine, appreciable or consistent differences in thyroxine binding by TBG, as compared with those found during the control state, could not be discerned; the small radioactive peak seen in Figure 2 was not found in other samples obtained during the same treatment period. The thyroxine-binding capacity of TBPA was slightly reduced during administration of diethylstilbestrol, but was unaffected by treatment with prednisolone and L-thyroxine.

Red blood cell uptakes of labeled hormones (*Table I*). *In vitro* uptakes of labeled T_4 and T_3 from the patient's plasma by red blood cells were greatly increased in the basal state and were not appreciably altered during the several treatment periods. The red blood cell uptake of labeled triac was normal during both the basal state and the administration of diethylstilbestrol, but underwent a pronounced increase during the immediate postoperative period.

Thyroxine metabolism in vivo (*Table I*). The peripheral metabolism of I^{131}-labeled T_4, measured while the patient was in the basal state, was grossly abnormal (9, 13). Fractional rate of turnover of administered hormone was rapid, 23.1 per cent per day. The T_4 distribution space was moderately increased when related to total body weight (0.22 L per kg), and hormonal clearance

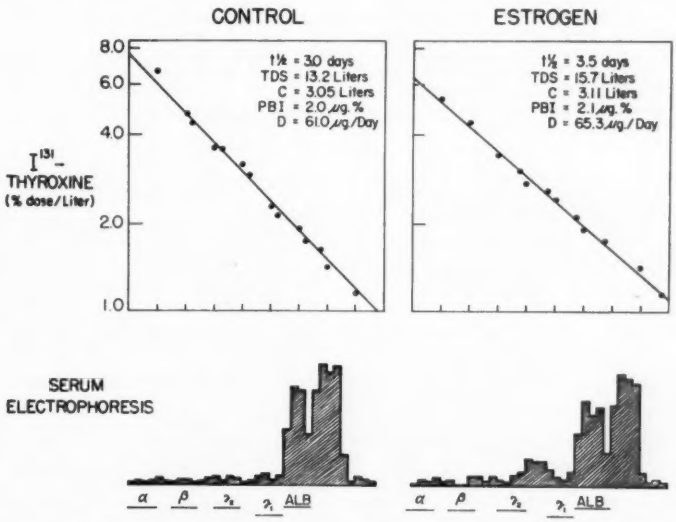

FIG. 2. THE EFFECT OF DIETHYLSTILBESTROL ON THE PROTEIN BINDING AND PERIPHERAL TURNOVER OF THYROXINE IN A PATIENT WITH DECREASED TBG. Thyroxine turnover studies during estrogenic therapy begun on Day 41 of treatment with 30 mg of diethylstilbestrol daily, p.o. Distribution of I^{131}-labeled thyroxine (0.4 µg per 100 ml) in sera obtained during each thyroxine turnover study and subjected to electrophoresis in Tris-maleate buffer, pH 8.6. The small peak of radioactivity seen in the TBG zone during estrogenic therapy was not evident in other samples obtained during the same treatment period.

TABLE I

Values for several aspects of thyroid hormone economy in normal subjects and in a patient with idiopathic decrease in thyroxine-binding globulin *

Function	Normal values†	Patient F.B.				
		Control	Diethyl-stilbestrol	Predni-solone	Postop-erative	L-thyroxine
Thyroid I^{131} uptake (*% dose*)	20–50	28	26			6
Basal metabolic rate	−15–+15	+3	+6	−1		+4
Serum cholesterol (*mg %*)	150–270	210	204			198
Protein-bound iodine (*μg %*)	4.0–8.0	2.0	2.1	2.1	1.8	2.3
Thyroxine turnover studies (9)						
Thyroxine space (TDS; *L*)	9.4 ± 2.0	13.2	15.7			
Turnover rate (k; *%/day*)	10.6 ± 0.9	23.1	19.9			
Clearance rate (C; *L/day*)	1.0 ± 0.3	3.05	3.11			
Organic iodine pool (ETT; *μg I*)	508 ± 146	264	329			
Daily hormone disposal (D; *μg I/day*)	53.6 ± 16.5	61.0	65.3			
Fecal fraction (F_{max}; *% dose*)	26.9 ± 9.2	25.7	27.2			
Urinary fraction (U_{max}; *% dose*)	73.1 ± 9.3	74.3	72.8			
Fecal clearance (*L/day*)	0.27 ± 0.11	0.78	0.85			
Degradative clearance (*L/day*)	0.73 ± 0.21	2.27	2.26			
Fecal excretion (*μg I/day*)	14.6 ± 6.8	15.6	17.8			
Degradation rate (*μg I/day*)	39.0 ± 12.7	45.3	47.5			
Hormonal binding studies (2, 36)						
TBG capacity (*μg thyroxine/100 ml*)	18–25	±‡	±	±	±	±
TBPA capacity (*μg thyroxine/100 ml*)	90–160	136	85	120	18	121
RBC thyroxine uptake (%)	0.6–0.9	1.5	1.5		1.7	
RBC triiodothyronine uptake (%)	5.0–8.5	13.2	12.8		14.4	
RBC triiodothyroacetic acid uptake (%)	1.2–2.1	1.5	1.9		2.9	

* For a description of functions and abbreviations shown under thyroxine turnover studies, see Appendix. For details regarding the several treatment periods, see text.

† Values presented are normal range or mean ± standard deviation. Normal values are those presented in previous studies or calculated from data contained therein, and were obtained by methods similar to those employed in Patient F.B. Numbers in parentheses provide references to these sources.

‡ ± Indicates traces of TBG inconstantly seen, and, when demonstrable, present in concentrations too low to permit calculation of thyroxine-binding capacity.

rate was greatly augmented (3.05 L per day). Because of the subnormal PBI, however, total hormonal disposal, 61.0 μg of iodine per day, was within normal limits. The extrathyroidal organic iodine pool was greatly reduced.

Radioiodine appeared in the urine more rapidly than normal after administration of labeled thyroxine. Butanol extractions of urine on the third, fourth, and fifth daily collections revealed that 3.3, 4.6, and 4.0 per cent, respectively, of urinary I^{131} was soluble in butanol and relatively insoluble in aqueous alkali; these values were similar to those found in normal individuals (14). Calculated values for the ultimate urinary and fecal excretion of I^{131} were 74.3 and 25.7 per cent of the administered dose, respectively. Both fecal clearance (0.78 L per day) and degradative clearance of T_4 (2.27 L per day) were greatly increased, but the daily fecal excretion rate and daily degradation rate were within normal limits.

Neither the peripheral metabolism of T_4 nor the concentration of hormonal iodine in the plasma was significantly altered by the prolonged administration of large doses of diethylstilbestrol. Neither prednisolone nor thyroid-suppressing doses of sodium-L-thyroxine increased the PBI.

DISCUSSION

There is general agreement that TBG serves as a rate-regulating factor in the peripheral metabolism of T_4. TBG, like other proteins with which T_4 is associated, is thought to limit the concentration of free or unbound hormone, thereby retarding its cellular penetration and decreasing the proportion of extrathyroidal hormone that is affixed to the cell. If, then, a constant proportion of the hormone within cellular confines is metabolized per unit time, the fractional rate of disposal of the entire peripheral pool of hormone will vary inversely with the binding activity of TBG (2, 3,

14). This hypothesis is largely based on inferential evidence, consisting of a series of correlations between changes in extracellular thyroxine-TBG interactions and changes in the metabolism of the hormone, either *in vitro* or *in vivo*. Thus, the uptake of T_4 by cellular systems *in vitro* varies inversely with the concentration of TBG and directly with the concentration of hormone in the suspending medium (15). *In vivo* correlations have mainly required the use of pharmacological agents to alter the thyroxine-binding capacity of TBG, associated effects on the peripheral metabolism of labeled T_4 being then determined. Diethylstilbestrol and natural estrogens, for example, increase the binding capacity of TBG (13, 16) and decrease the fractional rate of turnover of the hormone (13, 17), while methyltestosterone produces converse effects (18). These *in vivo* observations, while consistent with the major hypothesis, have not been entirely conclusive because of the possibility that the agents employed might directly affect cellular mechanisms for the disposal of hormone. This seemed especially possible in the case of diethylstilbestrol, in view of reports that this compound inhibits the deiodination of T_4 both by intact cellular preparations and by a partially purified thyroxine-deiodinase (19, 20).

More compelling evidence in favor of the postulated function of TBG has been afforded by the recently described patient in whose plasma there was found an apparently idiopathic increase in TBG (21). This abnormality was accompanied by changes in the peripheral metabolism of T_4 similar to those found in association with the increased TBG induced by estrogens (13). No gross evidence of endogenous excess of estrogens was evident in the male patient described. However, values for urinary estrogens were rather high and those for urinary gonadotropin rather low. These findings, together with the familial nature of the disorder, raised the possibility that some inherited disturbance in the secretion or metabolism of estrogens may have produced the increase in TBG which this patient manifested.

In the present patient, the apparently coincidental association of testicular hypofunction with virtual absence of TBG in the plasma afforded an unusual opportunity to complement existing data concerning the function of TBG. In view of the theoretical objections to the *in vivo* studies described above, it appeared desirable to obtain conclusive evidence that the abnormality in TBG displayed by this patient was not due to disturbances in known hormonal or metabolic factors which might, in themselves, influence the metabolism of T_4.

This patient's distinct hypogonadism excluded excessive secretion of normal androgens as the cause of decreased TBG. It appeared possible, however, that the patient's abnormal testes were secreting an unusual, nonandrogenic steroid capable of decreasing TBG. Apparently the capacity of certain steroids to decrease TBG is not a function of their androgenic potency, since norethandrolone,[10] a steroid with relatively more anabolic than androgenic activity (22), has proved capable of inducing pronounced reductions in TBG within a period as short as 1 week (23). During this time fractional peripheral turnover of T_4 was accelerated, but androgenic effects were not evident, even in hypogonadal patients (23). The large doses of diethylstilbestrol administered to the present patient produced gynecomastia and edema, suppressed gonadotropin production, and should have been adequate to suppress any lingering function that his testes may have retained. Nevertheless, diethylstilbestrol failed to restore the binding capacity of TBG to normal, and, indeed, any increase in TBG that may have been induced by this agent was too small to be recognized consistently during electrophoretic analyses.

Finally, it seems unlikely that an androgen-like steroid of adrenocortical origin was responsible for the decrease in TBG, since prolonged adrenocortical suppression failed to influence the abnormality in hormonal binding. The patient with decreased TBG reported by Tanaka and Starr had a history of hepatitis and displayed abnormal cephalin flocculation and thymol turbidity tests. In the present patient there was no clinical or laboratory evidence of hepatic or renal disease. Therefore, currently recognized causes of decreased TBG appear to have been excluded in the present case.

The abnormalities in the peripheral metabolism of T_4 which accompanied the decrease in TBG in this patient were consonant with the postulated function of TBG. The greatly increased red

[10] 17α-ethyl-17-hydroxynorandrostenone.

blood cell uptakes from the patient's plasma of T_4 and T_3, hormones bound primarily by TBG (14), provided evidence that an abnormally high proportion of these hormones was unbound or "free." As would therefore be expected, the T_4 distribution space was moderately increased *in vivo* and the fractional turnover of hormone was greatly accelerated. Accordingly, total hormonal clearance rate was markedly increased. However, the PBI was abnormally low, with the result that total hormonal disposal was well within the normal range.

Studies of urinary I^{131} and estimates of fecal excretion made possible an assessment of the general metabolic pathways by which the increased fractional rate of turnover was effected. Although fecal clearance of T_4 was increased several-fold, a finding in accord with earlier evidence that protein binding of T_4 limits its gastrointestinal excretion (24, 25), this increase accounted for only a small fraction of the increase in total T_4 clearance which the patient displayed. In the presence of an increased proportion of unbound hormone in the plasma, an increased renal clearance of T_4 would also be expected (14). Normally, however, this route of disposal contributes so little to total hormonal clearance that an enormous increase in this function would have been required to contribute significantly to the abnormality in hormonal turnover (26, 27). Measurements of the butanol-extractable I^{131} in the patient's urine confirmed the relative unimportance of urinary excretion of T_4 as a factor in the urinary excretion of I^{131} and in total hormonal disposal. Thus, increased hormonal turnover was due, in greatest part, to increased disposal of T_4 by those metabolic pathways which ultimately lead to hormonal deiodination.

It seemed important to exclude the possibility that this patient's subnormal PBI was not due to the abnormality in hormonal binding, but rather reflected the secretion of some thyroid hormone, like T_3, which, in amounts sufficient to maintain a normal metabolic state, does not contribute greatly to the PBI (28). If this were the case, then the metabolism of labeled T_4 would not have accurately reflected the metabolism of endogenous hormone. However, if T_3 were indeed the major hormone in the patient's plasma, then doses of L-thyroxine adequate to suppress endogenous thyroid function should have restored the PBI to normal. Since they did not, it seems likely in the present patient, as is normally the case (29), that T_4 constituted the major secretory product of the thyroid gland and the major constituent of the PBI. Calculated values for hormonal disposal were therefore validated.

The present association of normal thyroid function and metabolic status with normal values for daily T_4 disposal and deiodination, despite a subnormal PBI, supplements previous evidence, reviewed elsewhere (30), that both the secretion of thyrotropin and the metabolic state of the patient are more closely related to the quantity of hormone available to the tissues than to the total concentration at which they are delivered. The concentration of unbound hormone in the plasma, could it be readily determined, would likely constitute a better index of metabolic state than does the total PBI.

The failure of diethylstilbestrol to alter the kinetics of T_4 metabolism in this patient indicates that, in man, in contradistinction to certain cellular preparations *in vitro*, this agent apparently does not directly affect the cellular degradation of T_4. It therefore follows that the changes in T_4 metabolism that accompany the administration of estrogen to patients with normal TBG are indeed due to the resulting increases in TBG. Furthermore, the present findings support the belief that the changes in T_4 metabolism induced by androgenic hormones are not due to a direct cellular effect of the androgens, but rather to the associated decrease in TBG. Thus, the data lend considerable weight, both directly and indirectly, to the postulated function of TBG, described above. They do not, however, conclusively prove the hypothesis. Alternative interpretations of the function of TBG in the light of the present and previous observations concerning the metabolism of T_4 may also be envisioned (2). Furthermore, one cannot logically exclude the possibilty that the abnormalities in T_4 metabolism which this patient displayed arose, not from the decreased TBG, but from unrelated abnormalities in the cellular metabolism of the hormone.

In the absence of significant hormonal binding by TBG, an unusually large share of the transport of T_4 must devolve upon other proteins, notably TBPA (31). However, a consideration of the

possible physiological role of TBPA in the present patient, or indeed, in general, is beyond the scope of this report. While, in a number of circumstances, correlated changes in thyroxine binding by TBPA and in T_4 metabolism indicate that this protein contributes significantly to hormonal binding *in vivo* (2, 12), other evidence suggests that it may not (32, 33). Nevertheless, hormonal binding by TBPA seems adequate to explain several phenomena observed in the present studies. Thus, the normal values for thyroxine binding by TBPA found in the patient's serum when he was in a basal state correlated well with normal values for the red blood cell uptake of triac, since this derivative is primarily bound to TBPA, and not to TBG (11, 12). Similarly, the increased red blood cell uptake of triac obtained when the patient was in the post-operative period is probably explained by the reduced hormonal binding by TBPA which occurred at that time, a change similar to that found previously in sera from a large number of patients with other nonthyroidal illnesses (34). Subsequent studies in a large number of patients have indicated that the *in vitro* uptake of deaminated derivatives of T_4 and T_3 by an adsorptive particulate system varies inversely with, and is a convenient means of estimating, the thyroxine-binding capacity of TBPA (35).

As was the case in the patient reported by Tanaka and Starr (4), the defect in TBG in the present patient was associated with no gross abnormalities in the concentration of the major electrophoretically distinguishable groups of plasma proteins. Of the many transport functions of the plasma proteins only the transport of iron was assessed, and this proved to be normal. Thus, it is uncertain whether the abnormality described here is an isolated defect. In neither patient with this disorder has it been possible to ascertain whether the decrease in TBG was congenital or acquired, or whether it represented an inherited abnormality. The male patient with absent TBG described by Tanaka and Starr had no evidence of cardiac disease (4). Consequently, it is uncertain whether the congenital cardiac lesion in the present patient with decreased TBG represents a related abnormality or merely a fortuitous association.

Although the defect in the present patient has been referred to as a decrease in TBG, the concentration of the protein itself was not measured. Conceivably, decreased hormonal binding could have resulted from the presence of a highly effective inhibitor of thyroxine binding or from a minor structural abnormality in the protein that impaired the attachment of T_4. More likely, however, the abnormality described in this paper represents, as in other dysproteinemias (36), a decrease in the concentration of the protein itself. Whether, if this be true, the virtual absence of TBG from this patient's plasma is due to excessive destruction or defective protein synthesis, as is the case in agammaglobulinemia and analbuminemia (36), can not be determined at present. Resolution of these and other intriguing questions concerning the function and metabolism of TBG await ample supplies of the purified protein.

SUMMARY

1. Clinical features have been described of a euthyroid male patient with postoperative primary hypogonadism and a congenital atrial septal defect, whose plasma was virtually devoid of thyroxine binding by the thyroxine-binding globulin (TBG).

2. Prolonged administration of large doses of synthetic estrogen or adrenal corticosteroid failed to alter significantly the binding activity of TBG, suggesting that the abnormality in this protein was not produced by an androgen of testicular or adrenocortical origin.

3. This seemingly idiopathic decrease in TBG was associated with pronounced abnormalities in the peripheral metabolism of thyroxine. Both the volume of distribution and fractional rate of turnover of I^{131}-labeled thyroxine were increased; hormonal clearance rate was markedly augmented. However, the serum protein-bound iodine was subnormal, with the result that daily disposal of hormone by both excretory and degradative routes was within normal limits.

4. In contrast to its action in normal individuals, in whom it induces an increased binding activity of TBG, estrogenic therapy had no significant effect upon the concentration or peripheral turnover of thyroxine in this patient.

5. The several findings in this patient are viewed as consistent with the hypothesis that TBG regulates the peripheral metabolism of thyroxine by

limiting its cellular penetration and hence its peripheral metabolism and action.

APPENDIX

In this manuscript and Table I the following terms have been employed. These were described in detail in an earlier communication (9).

TDS, thyroxine distribution space; the virtual volume of body fluids through which exchangeable thyroxine would be distributed were it present throughout at the same concentration at which it exists in the plasma.

PBI, concentration of hormonal iodine in the plasma, and, by definition, the geometrical mean concentration of hormonal iodine in the TDS; assumed to represent solely iodine in thyroxine.

ETT, extrathyroidal thyroxine or the organic iodine pool; the total quantity of thyroxine within the TDS, in terms of its content of iodine.

k, fractional rate of turnover of thyroxine; the proportion of ETT removed from the TDS per unit time.

$T_{\frac{1}{2}}$, thyroxine half-time; the time required for one-half of the ETT to be removed from the TDS.

C, thyroxine clearance rate; the volume of plasma containing an amount of thyroxine equal to that removed from the TDS per unit time. Defined as "volume turnover, V" in an earlier communication (9).

D, hormonal disposal rate; the quantity of hormone removed from the TDS per unit time, in terms of its content of iodine.

F_{max}, the proportion of D accounted for by fecal excretion of hormone; U_{max}, the proportion of D accounted for by urinary excretion of organic iodine and iodide released during hormonal deiodination; fecal clearance, the volume of plasma that contains a quantity of hormone equal to that lost in the feces per unit time; degradative clearance, the volume of plasma containing a quantity of hormone equal to that removed by nonexcretory pathways per unit time. As calculated, degradative clearance includes the renal clearance of thyroxine, but the latter comprises a negligible proportion of the total; fecal excretion, the quantity of hormone excreted in the feces per unit time; degradation rate, the quantity of hormone removed by nonexcretory pathways per unit time.

$ETT = PBI \times TDS$. $C = TDS \times k$. $D = C \times PBI = ETT \times k$. $T_{\frac{1}{2}} = 0.693/k$. Fecal clearance $= C \times F_{max}$. Degradative clearance $= C -$ fecal clearance $= C \times U_{max}$. Fecal excretion = fecal clearance $\times$ PBI $= D \times F_{max}$. Degradation rate = degradative clearance $\times$ PBI $= D -$ fecal excretion.

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OBSERVATIONS ON BACTERIOPHAGE TYPING OF *PSEUDOMONAS AERUGINOSA*

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Recent reports indicate that there have been increasing numbers of serious infections due to *Pseudomonas aeruginosa* during the past few years (1–3). This has also been true at this hospital where the number of cases of bacteremic infection with this organism and of deaths in such cases has been steadily increasing since the introduction of potent antibacterial agents (4). *Ps. aeruginosa* has become one of the most important infectious agents, rivaling the staphylococcus as a cause of septicemia and death in patients with burns (5–7); it is being found frequently in infected surgical wounds (8), and outbreaks of serious and fatal infections with this organism have been reported in nurseries (9). It causes notoriously intractable urinary tract infections (10, 11), and it is being recognized as a major cause of serious and fatal superinfections in patients under intensive treatment with multiple or broad spectrum antibiotics (12–14). Most recently it has proved to be a major cause of bacteremia and death in patients under treatment for staphylococcal infections with methicillin (dimethoxyphenyl penicillin) (15–17). Most of the pseudomonas infections seem to arise in hospitals (12, 18, 19) and some studies have implicated inhalation of humid air (20–22) and contaminated water (23) as sources of isolated outbreaks in nurseries, but the sources of most of the sporadic infections within hospitals remain obscure.

The use of bacteriophage typing for the characterization of strains within certain genera of bacteria is now well known and has had many applications (24). This method, applied to strains of *Staphylococcus aureus* has proved particularly useful in recent years for the identification of sources of endemic and epidemic spread of infections with this organism, particularly in nurseries and surgical operating rooms (25–27). However, although phages of *Ps. aeruginosa* have been recognized and studied in some detail in various laboratories, and they have been used to some extent for the classification of strains of this species and in attempts to distinguish strains from various sources, they have not been widely used for "typing" strains or in the epidemiology of pseudomonas infections in hospitals. For these reasons it seemed appropriate to explore the possible uses of pseudomonas phages for such purposes.

The present paper deals with the isolation of pseudomonas phages from clinical sources, some observations on their properties, and a preliminary exploration of the application of these and some other available phages to the typing of strains of *Ps. aeruginosa* from clinical infections. Soon after this study got under way, Gould and McLeod (28) reported in detail on their study, based on bacteriophage and serological techniques, of pseudomonas infections in hospitals. Their report, which contains the pertinent references to previous work bearing directly on this subject, has proved most valuable in the conduct of the present study.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. Seven phages and their propagating strains (PS) were obtained from the American Type Culture Collection ATCC (29). The phages had been designated 12055-B1, -B2, and -B3; and 12175-B1, -B2, -B3, and -B4; the propagating strain of *Ps. aeruginosa* for the first three phages was ATCC-12055,[1] and for the other four it was ATCC-12175. All other strains of *Ps. aeruginosa* were isolated in the bacteriology laboratory of the Boston City Hospital and all other phages were developed from these strains in the course of this study.

The following criteria were used for identification of *Ps. aeruginosa:* motile, gram-negative rods; typical colonial morphology on 10 per cent sheep's blood agar and on eosin methylene blue agar; for most, but not all,

* Aided by Grant E-23 from the National Institutes of Health.

[1] This is host C10 of Dickinson and Codd (30, 31).

strains a diffusible green, or, in a few, a reddish-brown pigment on trypticase soybean agar; pellicle formation in liquid media; growth at 30 to 42° C; glucose the only one of the commonly-used sugars fermented; and characteristic odor. In a previous study of pseudomonas strains reported from this laboratory (32), the formation of a green or brown color on Gessard's glycerol agar was found to be the most reliable test for the production of pyocyanin; by that criterion 103 of 110 strains were classified as *Ps. aeruginosa* and 7 were considered to be atypical strains of pseudomonas, since they failed to produce the pigment. No such differentiation was attempted in the present study.

In all, 161 strains of *Ps. aeruginosa* were isolated and used in this study. Most of them were obtained from cultures of urine or infected wounds, some from blood, peritoneal exudate or cerebrospinal fluid, and occasional ones from specimens of feces that were negative for enteric pathogens and in which the predominating coliform bacilli had first been suppressed by growth in liquid tetrathionate medium.

Isolation, propagation and characterization of Ps. aeruginosa bacteriophages. The procedures were similar in many respects to those used by Gould and McLeod (28). The media used routinely were: trypticase soy broth [2] (TSB), 1.5 per cent trypticase soy agar [2] (TSA), and a semisolid medium consisting of equal parts TSB and TSA.

Each of the phages obtained from ATCC was propagated as follows: 1 ml of a 5-hour TSB culture of the propagating strain was flooded on a TSA plate and the excess fluid removed. The plate was placed in an incubator at 37° C with the lid tilted for 25 minutes to permit drying, after which 1 ml of concentrated phage suspension containing 10^8 to 10^9 phage particles was poured over the surface and permitted to dry again in the same manner after removing the excess fluid. The plate was covered and incubated overnight (16 to 18 hours) at 31° C. The surface of the plate then appeared to be largely free of the bacterial lawn and had only isolated colonies, which usually represented phage-resistant or lysogenic mutants. The "bare" plate was refrigerated at −20° to −30° C for 24 to 72 hours and then thawed at room temperature, yielding varying amounts of clear fluid containing concentrated phage, which was then harvested as the clear supernatant after centrifugation at 3,000 rpm for 30 to 60 minutes. The phage suspension was heated at 58° to 60° C for 2 hours to kill any residual bacterial cells. This procedure did not affect the titer of phage appreciably, thus confirming the observations of Dickinson (33).

The titer of phage in the suspension was estimated by plaque counting (24) and also roughly by delivering single drops of 10-fold dilutions of the suspension with a Pasteur pipet on a dried lawn of the propagating strain. The plaque-counting method also permitted visualization of plaque morphology. The routine test dilution (RTD) for that phage suspension was then selected. This differed from that employed in phage typing of staphylococci (34) in that 10 or, more often, 100 times the highest dilution giving confluent lysis was used as the RTD for the present study in order to permit visualization of various types of lysis produced by the same phage on different strains; this also served to reduce to some extent the variations in the degrees of susceptibility of different strains of *Ps. aeruginosa* to any given phage. The RTD for most of the phages was a 10^{-2} dilution of the phage suspension, but it ranged from 10^{-1} to 10^{-4} (equivalent to about 10^6 to 10^9 or more phage particles per ml by the plaque-counting method).

In the isolation of new phages, advantage was taken of the spontaneous lysis observed in some cultures of *Ps. aeruginosa* on solid media as evidenced by the moth-eaten appearance of individual colonies, probably representing an unstable lysogenic state of that culture (temperate phage).[3] An isolated moth-eaten colony together with its base agar was cut out and put into TSB, which was then refrigerated for 48 or 72 hours at 4° C and centrifuged. The supernatant was removed, heated, and used as a phage suspension. A lawn of each strain of *Ps. aeruginosa* to be tested was then prepared on the surface of an agar plate and individual drops of the various phage suspensions were placed on this lawn. The susceptibility of the bacterial strain to these phages was evidenced by the development of clear plaques. Bacterial strains showing confluent lysis with any phage suspension were then chosen as indicator and propagating strain.[4] When a suitable host or indicator strain was not found in this manner, a bacterial lawn was flooded with the phage suspension and isolated clear plaques were then looked for as representing virulent phage. Phage mutants which had a wider host-range than their parent phages were sometimes derived in this manner. Each isolated plaque was then picked and placed in a growing 4-hour broth culture of the bacterial strain which had yielded the plaque. The mixture was incubated for only 6 hours, at which time the maximal yield of phage was obtained. The suspension of the lysed areas was then refrigerated and subsequently subjected to centrifugation, heating, titration, and propagation on agar plates as already described for the known phages.

The "phage typing" of strains of pseudomonas was carried out essentially as with staphylococci, using a lawn of a 5-hour TSB culture on "checkerboard" plates on which a drop of RTD (as defined above) of each of the phages was placed. The plates were read after overnight incubation. Clear, confluent lysis was read as +++, 20 to 30 clear plaques as ++, less than 20 clear plaques as +, and no lysis as −. Another type of reproducible lysis, best described as "turbid plaque" formation was also re-

[2] Obtained from Baltimore Biological Laboratories.

[3] The "metallic spots" sometimes noted in pseudomonas cultures are morphologically different and probably do not represent a phage phenomenon (35).

[4] An "indicator" bacterial strain is one that is lysed by a particular phage but not by some others; a "propogating" strain is one that is maintained and used to prepare a stock of a particular phage (and sometimes more than one) to which it is susceptible.

corded and designated by the addition of "T" after the phage designation. The type or pattern of a pseudomonas strain was then represented by the designation of the phages which produced confluent, that is, +++ or +++ T lysis.

The visualization and counting of plaques was done with double-layered TSA plates. The base layer contained 10 ml TSA (1.5 per cent), and the surface layer consisted of 2 ml of semisolid agar (0.75 per cent TSA) containing 0.1 ml of the desired dilution of phage suspension and 0.1 ml of a 5-hour TSB culture of the propagating strain. The soft agar hardened promptly after being delivered over the previously hardened base layer. The number and morphology of the plaques were recorded after overnight incubation.

Bacterial mutants resistant to a particular phage were developed from a susceptible bacterial strain by flooding a suspension of phage on a lawn of the culture and allowing it to grow overnight. Individual phage-resistant colonies were picked into TSB and grown overnight at 37° C. The resulting culture was washed in TSB, diluted, and plated so as to produce individual colonies. A culture from a single colony was retyped and then used as an indicator strain in the plaque visualization and counting procedure for the phage in question in order to ascertain the nature and degree of the resistance of the mutant strain to that phage.

RESULTS

Development of the set of typing phages. Three criteria were used in selecting phages for inclusion in typing set: *1*) host range, as determined by exposure of the 161 isolates from this hospital and the 2 host strains obtained from ATCC; *2*) development of resistance to individual phages and tests of cross resistance to other phages; and *3*) visualization of plaque morphology. When two or more preparations of phage suspensions derived from different sources behaved in identical manner with respect to these three criteria, only one of them was chosen for inclusion in the set together with the propagating strain that gave the best yield of that phage. The 13 distinct phages thus chosen are listed in Table I, which also lists the relevant data concerning their sources as well as those of their respective propagating strains. The phages were divided into four groups, based on similarities with respect to one or more of the three criteria. The lytic patterns produced by these phages on all of the propagating strains are shown in Table II.

Phage typing of clinical isolates of Ps. aeruginosa. The set of 13 phages thus selected was used in the "phage typing" of 161 isolates of *Ps. aeruginosa* obtained from various clinical sources throughout the hospital. The largest number, 49, were from infected urines; 25 from infected wounds; 22 from sputum or tracheal aspirates; 14 from cultures of feces which, as already noted, had been found negative for enteric pathogens and from which the predominant coliform organisms had been eliminated by cultivation in tetrathionate

TABLE I

The set of Pseudomonas aeruginosa typing phages

Phage			Propagating strain			Yield, plaques per ml	Plaque morphology†	
Group	Type	Origin*	No. (PS)	Strain of origin	Source		Size	Edges¶
A	1	Phage ATCC-12055B_1	1	BCH-12	Sputum	10^8	Small	Irreg. [5]
	2	Phage ATCC-12055B_1M	2	BCH-47a	Urine	10^8	Very large	Sharp.
	3	BCH-165a (ear)	3	ATCC-12175m	Mutant‡	10^{10}	Very large	Irreg. [1]
	4	ATCC-12175B_1	4–5	ATCC-12175		10^{10}	Large	Sharp
B	5	ATCC-12175B_2	4–5	ATCC-12175		10^{10}	Large	Sharp
	6	BCH-15 (wound)	6	BCH-29	Urine	10^8	Medium	Sharp
	7	BCH-79 (urine)	7	BCH-129 brown	Urine	10^7	Variable	
C	8	BCH-5 (blood)	8	BCH-30	Urine	10^8	Medium	Sharp
	9	BCH-94 (urine)	9	BCH-88	Feces	10^{10}	Variable	
	10	BCH-94§ (urine)	10	BCH-137	Urine	10^{10}	Medium	Irreg. [2]
	11	BCH-137 (urine)	11	BCH-127	Sputum	10^9	Medium	Irreg. [4]
D	12	ATCC-12055B_1	12–13	ATCC-12055		10^9	Medium	Irreg.
	13	ATCC-12055B_2M‖	12–13	ATCC-12055		10^9	Medium	Irreg.

* Source of phage or original designation of the lysogenic donor strain of *Ps. aeruginosa*, the source of which is given in parentheses.
† All plaques were clear.
¶ See Figure [ ].
‡ Mutant bacterial strain selected for resistance to phage 12175B_2 (which is here designated as phage 5).
§ Mutant phage isolated from the same lysogenic strain of *Ps. aeruginosa* that yielded phage 9.
‖ This mutant phage produced clear confluent lysis.

TABLE II

Pattern of lysis of propagating strains (PS) by the set of typing phages

No. (PS)	Phages													Phage pattern*
	A				B			C				D		
	1	2	3	4	5	6	7	8	9	10	11	12	13	
1	+++	+	±	±	−	−	−	−	−	−	−	−	−	A(1)
2	+	+++	−	−	+	−	−	−	−	−	−	−	−	A(2)
3	+++T†	+++T	+++	+++	±	−	−	−	−	−	−	−	−	A(3/4)
4–5	+++T	+++T	+++	+++	+++	+++	−	−	−	−	−	−	−	A/B(3/4/5/6)
6	+++T	+	++	++	+	+++	−	−	−	−	−	−	−	B(6)
7	−	−	−	−	−	−	+++	−	−	−	−	−	−	B(7)
8	−	−	−	−	+++	+++	−	+++	−	−	−	−	−	B/C(5/6/8)
9	−	−	−	−	−	−	−	+++	+++	+++	−	−	−	C(8/9/10)
10	−	−	−	−	−	−	−	−	+	+++	−	−	−	C(10)
11	−	+++T	−	−	−	−	−	−	−	−	+++	−	−	C(11)
12–13	+	−	−	+	−	−	−	−	−	−	−	+++	+++	D(12/13)

* Pattern based only on confluent (+++), complete (clear) lysis.
† T = turbid or hazy lysis, not listed in the phage pattern.

broth; 10 from otitic exudate; 6 from blood cultures; and the rest from other or unknown sources. The latter included 20 strains that had been isolated 18 or more months previously for different studies and had been kept frozen, whereas all of the others were recent isolates made after this study began.

The strains were then classified according to the patterns of lysis with RTD of the different phages, separating those on which the lysis was confluent and clear from those yielding only turbid lysis. In every instance in which confluent lysis, clear or turbid, was produced with RTD (as here defined) of any given phage, the same type of lysis was reproduced on subcultures of the same strain with multiples of RTD. Both the clear and turbid lyses were reproduced on different occasions at least once, and the latter was reproduced numerous times with two strains. Moreover, the same pattern of both kinds of lysis was reproduced whenever multiple isolates of *Ps. aeruginosa* were obtained from the same or different sources in the same patient. A third type of lysis was observed with phage 7 when it was applied undiluted on certain strains. This was an irregular pattern of lysis with fuzzy edges which was also reproducible with subcultures of the same strains.

The results of the phage typing of the 161 isolates are summarized in Table III, which also lists the sources of the strains of each phage pattern. From part A of this table it is seen that 78 strains (48.4 per cent) could be typed on the basis of the patterns of clear and confluent lysis, and that 19 phage patterns of such lysis were discerned among the 78 strains. Included among the 78 were 2 to 4 isolates obtained from the same or different sources in each of seven patients. In each of these patients all of the isolates produced the identical phage pattern; in three this was B(5/6); in three others it was B(5/7); and the isolates from the seventh patient all typed at B(7), each producing the same type of reddish-brown pigment. The proportion of strains from each source (i.e., from urines, wounds, and so forth) that could be typed on the basis of clear lysis did not differ markedly from that for the total of all strains except that a somewhat smaller proportion of those of miscellaneous or unknown sources could be typed in this manner. Of the 29 strains in the latter category, only 9 (31 per cent) produced patterns of clear lysis.

Part B of Table III lists the number of isolates from different sources that failed to produce confluent clear lysis but which yielded reproducible patterns of uniformly turbid lysis with RTD of some of these typing phages. There were 50 such strains and it is interesting to note that all of them reacted only with RTD of the four phages classified here as group A. Also listed as of the last phage pattern in part B of Table III are 16 strains that yielded irregular plaques of turbid lysis with fuzzy edges only with the undiluted suspension of phage 7. A total of 65 strains (40.4 per cent) could be typed in this manner, and they fell into nine patterns, including B(7T). Multiple isolates of *Ps. aeruginosa* were obtained at different times from the same or different sources in four patients; here again all isolates from any one patient yielded the same phage pattern.

In all therefore, 143, or 88.8 per cent, of the 161

TABLE III

"Phage patterns" and sources of 161 clinical isolates of Ps. aeruginosa

Phage pattern	Number of strains isolated from: Infected urine	Infected wounds	Sputum or tracheal exudate	Feces[a]	Otitic exudate	Blood culture	Deep abscess	Others, or unknown source	All sources
A. Patterns of confluent clear lysis with RTD									
A(1)			1						1
A(1/2)		1							1
A(2)	2								2
A(2/3/4)	1								1
B(5)	1			1					2
B(5/6)	3	6	2		3	1	1	5	21[b]
B(5/7)	6	1	6			2		3	18[c]
B(6)	2	1	3		1				7
B(7)	3[d]	1							4
B/C(5/8)				1					1
B/C(5/6/8)	1								1
B/C(7/8)		1							1
C(8)		3	1	1				1	6
C(9)	1	1							2
C(10)	1								1
C(11)			1						1
C(8/9/10)	2			4					6
C(8/9/10/11)				1					1
C(9/10)		1							1
Total	23	16	14	8	4	3	1	9	78
B. Patterns of confluent but turbid or hazy lysis with RTD									
A(1T)	2	2						1	5
A(1T/2T)	3	3	4	4	3		2[e]	1[f]	20[g]
A(1T/2T/3T)	1								1
A(1T/2T/4T)			1					4	5
A(1T/4T)	5	1						1	7
A(1T/2T/3T/4T)		1			1			4	6
A(2T)	1	1							2
A(4T)				1		1		1	3
B(7T)[h]	8	1	2			1	1	3	16[i]
Total	20	9	7	5	4	2	3	15	65
C. Nontypable (NT) with RTD or concentrated phages									
NT	6		1	1	2	1	2	5	18[j]
All strains	49	25	22	14	10	6	6	29	161

[a] All fecal strains were from specimens negative for enteric pathogens.

[b] Four separate isolates (3 from leg ulcer and 1 from urine) from one patient; 3 separate isolates (2 ear and 1 throat culture) from a second patient and 2 (both from wound) from a third.

[c] Two separate isolates from each of 3 patients (both from urine in one, both from sputum in the second and 1 from urine and the other from a blood culture in the third).

[d] All from separate urines obtained at different times from the same patient and each produced reddish-brown pigment.

[e] One from osteomyelitis, 1 from empyema.

[f] From cerebrospinal fluid.

[g] Two strains isolated from separate stools of same patient.

[h] Irregular plaques of turbid or hazy lysis produced only with undiluted phage.

[i] Four separate isolates from one patient (1 each from urine, nephrostomy drainage, blood culture and cerebrospinal fluid); 2 isolates from different urine specimens in a second patient and 2 isolates (one from sputum and the other from a burn wound) in a third.

[j] Two of these were separate isolates from an abdominal abscess in one patient and 2 others were from otitic exudate in a second patient.

TABLE IV

Susceptibility of parent and phage-resistant mutant strains of Ps. aeruginosa to the set of typing phages

Strain of *Ps. aeruginosa**	Phages													Phage pattern†
	A				B			C				D		
	1	2	3	4	5	6	7	8	9	10	11	12	13	
BCH-63 Parent	+++T	+++T	−	−	+++	−	+++	−	−	−	−	−	−	B(5/7)
Mutant‡	+++T	+++T	−	−	+++	−	−	−	−	−	−	−	−	B(5)
BCH-31 Parent	−	−	−	−	−	−	−	+++	+++	+++	−	−	−	C(8/9/10)
Mutant§	−	−	−	−	−	−	−	−	+++	+++	−	−	−	(C9/10)

* Another such pair of strains, shown in Table II, consists of PS4–5 (parent) and PS3 (mutant resistant to phages 5 and 6).
† Confluent, clear lysis.
‡ Resistant to phage 7.
§ Resistant to phage 8.

strains could be classified with these phages. The remaining 18 strains (11.2 per cent) failed to produce any lysis with any of the 13 phages of the typing set and were therefore classified as nontypable (NT), as shown in part C of Table III.

Not shown in Table III is the distribution of the patients from whom the strains were obtained among the different hospital services and wards. Actually, the strains of each of the phage patterns were obtained from many wards and there was no concentration of these patients in any given part of the hospital. Three was the largest number of patients with strains of the same phage pattern on any one ward; they were among the 15 patients from whom strains of B(5/6) were isolated. There were four wards in which two patients yielded strains of the same phage pattern, a different phage pattern being represented in each ward.[5]

Development of phage-resistant mutants from phage-susceptible bacterial strains. In the course of this study, isolated colonies of bacterial growth were sometimes observed within an area of otherwise clear and confluent lysis produced on the lawn of a strain of *Ps. aeruginosa* on which one of the phage suspensions had been placed. Some of these colonies were subcultured, washed free of residual phage and studied for their susceptibility to the phages of the typing set. The results thus obtained with two mutant bacterial strains, resistant to the respective phages by which they were recognized, with their respective parent strains are shown in Table IV. A third pair of such strains was included among the propagating strains of the typing set of phages, namely, the parent strain PS4–5 and the derived mutant PS3 isolated from the area of lysis of PS4–5 produced with phage 5.

From Table IV it is seen that the mutant bacterial strain derived from BCH-63 after it had been acted on by phage 7 gave a phage pattern identical with that of the parent strain except for its failure to be lysed by that phage. Likewise, the phage pattern of the mutant derived from BCH-31 when that strain reacted with phage 8 was identical with its parent except for its resistance to phage 8. From Table II it is seen that PS3 which was derived from a colony within the area of lysis produced on PS4–5 by phage 5 was partially resistant to phage 5 and completely resistant to phage 6, to which PS4–5 was fully sensitive.

Observations on the color produced by some strains of Ps. aeruginosa and their phage susceptibility. Phage 7 of the present typing set is characterized by a rather wide host range. Among the 161 clinical isolates, it produced clear and confluent lysis in RDT of 23 strains, and irregular and atypical lysis of 16 others when applied as the undiluted suspension, each type of lysis being reproducible in every instance. The action of phage 7 on the propagating strains for the typing set of phages, however, was highly specific, as shown in Table II. Only its own PS (BCH-129), which regularly produced a diffusible reddish-brown pigment on TSA, was fully susceptible to phage 7. The development of this phage is of interest.

Early in the present study it was noted that strains yielding a diffusible brownish-red or brown-to-purple color [probably due to pyorubrin (36)] were not susceptible to any of the phages originally obtained from ATCC. Only a phage derived

[5] These pairs of patients were, respectively, from among 7 patients with type B(6); 15 with B(5/7); 4 with C(8/9/10); and 19 with phage pattern A(1T/2T).

from the lysogenic yield of a clinical strain of *Ps. aeruginosa*, BCH-79, when propagated on another strain, BCH-63, produced a few plaques of lysis on three available strains that produced deep brown pigment, *viz.*, BCH-90, -98 and -129. These three isolates had all been obtained from cultures of separate urine specimens of one patient with chronic pyelonephritis. The plaques produced on BCH-129 were then propagated first in a growing broth culture of that bacterial strain, the heated supernatant was further propagated on TSA plates seeded with BCH-129 which was then selected as PS7, and the resulting phage was included in the typing set as phage 7.

The sequence of events and probable mechanisms involved were somewhat as follows. The original phage yield from the brown, lysogenic strain BCH-79, when propagated on the green strain BCH-63, contained a phage mutant with a wider host range which included the brown isolates BCH-90, -98 and -129. After propagation on the brown strain BCH-129, now PS7, the phage (now labeled phage 7) retained its wide host range irrespective of whether it was propagated on the green BCH-63 or the brown PS7. Furthermore, the concentrated suspension of phage 7 could lyse several strains of pseudomonas not susceptible to other phages and some of these strains yielded a diffusible purplish color on TSA, especially if the growth was allowed to stand for 1 or 2 days exposed to daylight. Three such strains were BCH-19, -20 and -27. However, although the concentrated phage 7 produced irregular lysis, and individual plaques of varying size could be demonstrated by the plaque-visualization method, repeated attempts to propagate phage from these plaques in growing cultures of strains BCH-19, -20 and -27 failed. It therefore seems highly probable that the irregular lysis was the result of a direct lethal effect of the large numbers of phage particles on the bacterial strains.

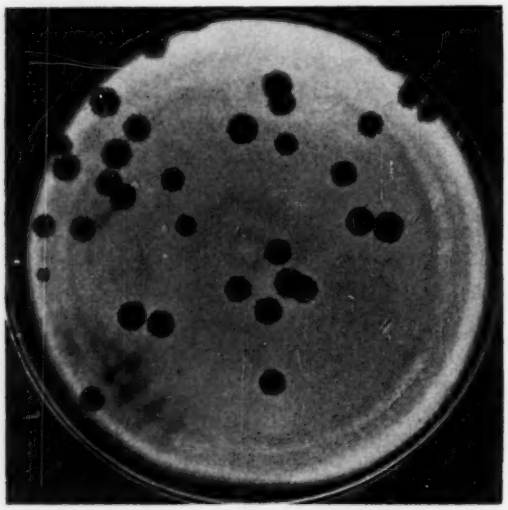

FIG. 1. DILUTION 10^{-8} OF PHAGE 3 ON PS3. The plaques are clear, very large and have irregular edges.

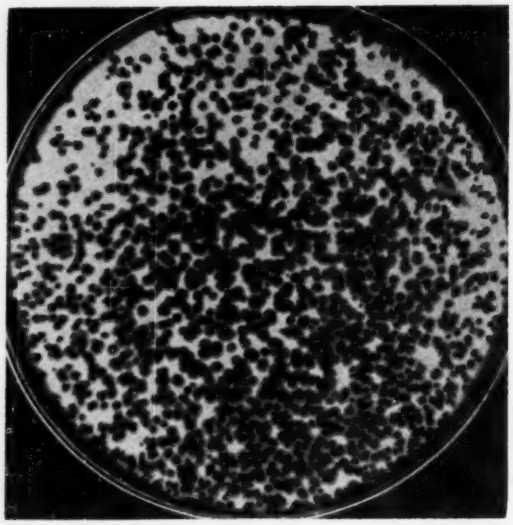

FIG. 2. DILUTION 10^{-7} OF PHAGE 10 ON PS10. The plaques are clear, of medium size, and sharply defined but of irregular outline.

The loss of color production frequently observed in some strains of *Ps. aeruginosa* (32, 37) makes the estimation of the yield of pigment an unstable genetic marker for these organisms. The ability of phage 7 to lyse both green and brown (red) strains is therefore of some interest.

Observations on plaque morphology. The final separation of phage clones was carried out by the plaque-counting technique which also permitted visualization of the morphology of the individual plaques. Figures 1 and 2 illustrate the appearance of the plaques of two of these phages.

The turbid [6] plaque was of particular interest and the subject of more detailed study because it

[6] The term is used here to describe the centeral lytic area of the plaque and does not refer to the peripheral zones of partial lysis, such as those shown in Figures 4 and 5.

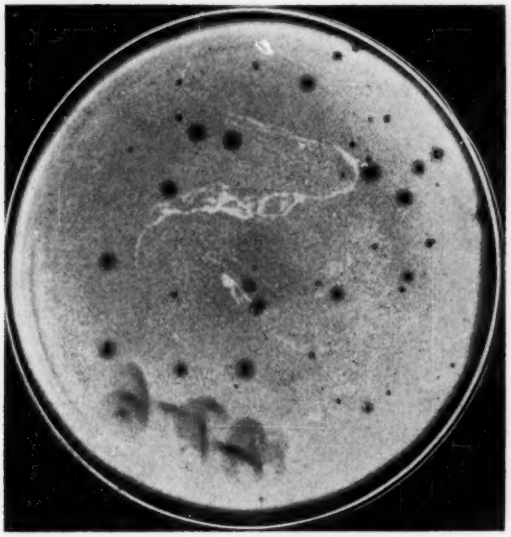

FIG. 3. DILUTION 10^{-7} OF PHAGE 11 AS ORIGINALLY ISOLATED FROM THE PHAGE YIELD OF BCH-127 PROPAGATED ON THAT STRAIN AS AN INDICATOR. Note the three types of plaques: clear center with irregular edges; turbid center within a clear area and irregular outer borders; very small clear plaques. A fourth type, small and diffusely turbid, was also present but not seen in the photograph.

was frequently employed, under special conditions, in the typing of strains of pseudomonas, as already noted. Several possibilities were considered to explain this variety of plaque morphology: *1*) mixed host bacterial culture; *2*) lysogenic variants or phage-resistant mutants selected out of the bacterial culture during exposure to a specific phage; *3*) a mixture of phages; *4*) development of host-range mutants of the phage; and *5*) temperate phage with lysogenization of part of the host culture exposed to the phage and lysis of the rest of the culture by virulent phage.

An example of an attempt to analyze the mechanism of turbid plaque formation may be cited from the experience with the early development of typing phage 11. The initial phage suspension from which this phage was derived produced a mixture of clear and turbid plaques on the indicator strain that was used, as shown in Figure 3. When one of the clear plaques was picked and propagated on the same host culture, it gave rise to clear plaques exclusively, as shown in Figure 4.

To rule out the possibility that a mixed culture accounted for the turbid plaque, PS11 was grown in TSB and then 10 indivdual colonies were picked from TSA pour plates of end-dilution of that culture. Each colony was grown separately in broth and then tested with the original phage suspension. Both clear and turbid plaques were produced on the cultures from each of the 10 colonies in a manner similar to that depicted in Figure 3. However, when purified phage suspensions were prepared by propagating single clear plaques on each of these single colony cultures, only clear plaques resulted, and these were always reproducible. Likewise, phage suspensions derived from single turbid plaques prepared in the same manner always yielded turbid plaques when propagated on each of the single colony cultures. This seems to indicate that the differences in plaque morphology were not the result of a mixed host culture.

When a drop of RTD of a suspension of phage prepared from one of the turbid plaques was used on a lawn of PS11, it produced confluent turbid lysis. A turbid section of agar was cut out of such a plate, inoculated into TSB, and a number of individual colony cultures prepared, as above. Each of the resulting cultures was then tested separately for susceptibility to the clear plaque-forming phage and to the phage prepared from the turbid plaque. All were found to be resistant to both varieties of phage. On the other hand,

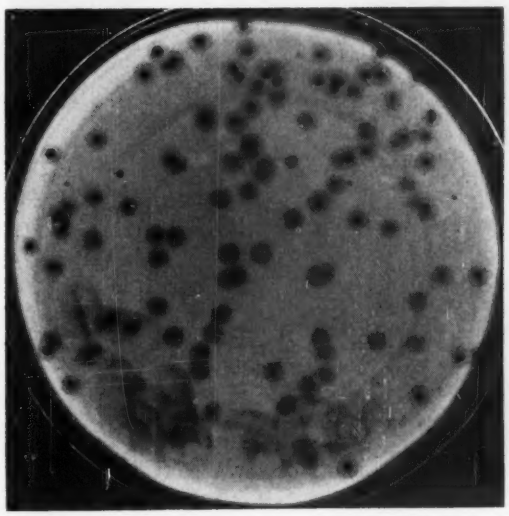

FIG. 4. DILUTION 10^{-6} OF THE CLEAR PLAQUE VARIANT SELECTED AS PHAGE 11 WHEN GROWN WITH PS11.

when the clear plaques were cultured in TSB, they yielded no growth, indicating the presence of virulent phage exclusively, which lysed all of the bacterial cells within the clear plaque.

This phenomenon of hazy plaque formation was noted by Dickinson (31) with her host strain C-10 of *Ps. pyocyanea* which served as PS for phages ATCC-12055-B_1, -B_2 and -B_3. She concluded that the clear plaque-forming phage was a virulent mutant of a predominantly temperate phage yield of a lysogenic strain of pseudomonas. The temperate phage, on the other hand, produced hazy plaques capable of establishing a lysogenic state in the host (indicator) strain.

Turbid plaques could also be produced artificially with a single virulent phage suspension screened against a background of a mixture of phage-sensitive and phage-resistant bacteria. Thus, when phage 7, which is lytic for *Ps. aeruginosa* strain BCH-63 was used to infect a bacterial lawn consisting of a mixture of BCH-63 and a phage 7-resistant mutant of that strain (Table IV), confluent turbid plaques resulted. Normally, phage 7 produced only clear plaques on the susceptible indicator strain, BCH-63, and had no effect on the phage 7-resistant mutant of that strain.

In the present study some of the typing phages

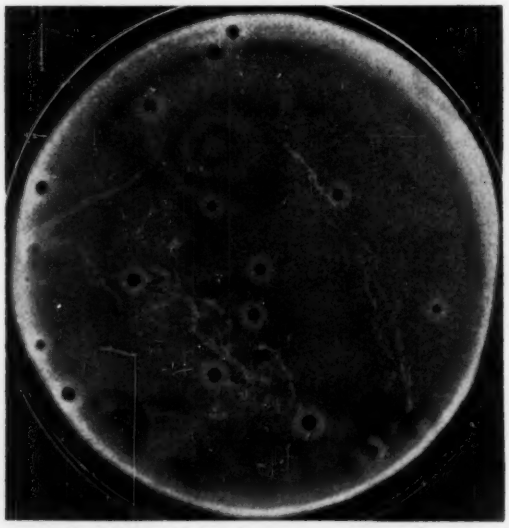

FIG. 5. DILUTION 10^{-5} OF PHAGE 1 ON PS1. Note the clear centers of the plaques and their irregular edges.

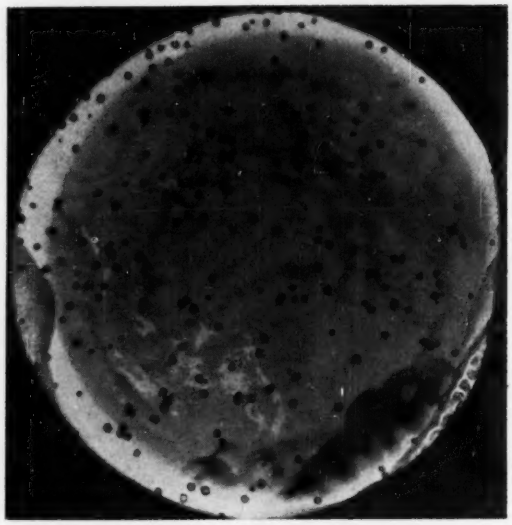

FIG. 6. DILUTION 10^{-6} OF PHAGE 1 ON INDICATOR STRAIN BCH-138. Note the turbid centers of the plaques and compare them with the clear plaques produced by this phage on PS1 (Fig. 5).

produced confluent clear plaques on their own propagating strains and on very few other strains, but they produced almost exclusively turbid plaques on all other susceptible bacterial host cultures. This was the case with phage 1 which produced clear lysis on PS1, as shown in Figure 5, and on only one other clinical strain of *Ps. aeruginosa,* whereas it produced only turbid plaques on a large number of hospital strains of pseudomonas (Table III) as illustrated in Figure 6. This is probably the result of the different degrees of virulence of phage 1 for the different bacterial strains. Phages 2, 3 and 4 also behaved in the same manner as phage 1, as can be seen from parts A and B of Table III. The frequent and reproducible finding of turbid plaque formation in the typing of clinical isolates of *Ps. aeruginosa* by the set of typing phages, when used in concentrations greater than the minimum that produced confluent lysis on the PS, was the reason for selecting RTD in the manner used in this study.

In the isolation of new phages, when a lysogenic bacterial strain serving as a phage donor was screened alone on semisolid agar, no plaques were visualized. This was to be expected, since only a certain proportion of the bacterial cells of the moth-eaten colony would lyse and yield phage to

which the remaining cells were presumably resistant. If the moth-eaten colony were incubated in broth, the phage-resistant bacteria would multiply without an increase in the yield of phage. For that reason, refrigeration at below freezing temperatures, followed by thawing of the suspension of the unstable lysogenic phage donor colony was used rather than incubation, as recommended by Gould and McLeod (28).

DISCUSSION

The present study should be considered only as an exploration of the possibility of utilizing bacteriophage for the classification of human pathogenic strains of *Ps. aeruginosa* and for the possible use of phage typing in the epidemiology of serious infections caused by this species. From that point of view the study seems to have been successful. It has been possible, with relative ease, to apply some of the basic phage technology and the rather limited previous data available on the application of bacteriophage to clinical strains of this organism, to establish a set of typing phages and to apply them in a manner somewhat similar to, but not identical with that used in phage typing of *Staphylococcus aureus*. Thus far the clinical virulence of the strains does not seem to be correlated with the phage types.

Most of the phages in the typing set were developed in the course of this study and only four of them were used directly from other sources or adapted from previously known phages. Those developed here have not yet been compared with those developed and used in other laboratories (28, 38–41). Moreover, it is recognized that phages of pseudomonas, as well demonstrated in this study, may be rather difficult to maintain in "pure" lines because of the frequency of lysogenization and the unstable lysogenic state of the bacterial hosts. However, the possibility of "purifying" the phages by the methods applied here seems feasible so that it is reasonable to look forward to a system of phage typing not much less satisfactory than that now available for *Staphylococcus aureus*.

Several favorable and promising aspects were brought out in this study. Among them is the fact that once the possibility of a variant or mixture of phage or culture was recognized, it proved feasible in most instances to select and purify the phage and its propagating strain so that they were again useful for typing purposes. Even more important is the fact that typing of any given clinical strain was reproducible with the same cultures and with subsequent subcultures. Moreover, in every instance in which multiple isolates of *Ps. aeruginosa* were made at the same time from different sources or at different times from the same or different sources in the same patient, all of them produced a phage pattern essentially identical with the typing set. This suggests that the organisms retain their specific pattern of phage susceptibility in any given patient, a property which is essential if typing is to be useful in clinical and epidemiological work. Finally, it is most encouraging that nearly 90 per cent of 161 strains isolated from clinical materials in this hospital over a period of several months could be classified with the typing set that was developed.

It remains to be determined to what extent the typing set can be maintained in reasonably stable form over long periods. It will also be necessary to determine how widely these phages can be applied in the identification of strains from other sources within this hospital and from patients and other sources in other hospitals or outside of hospitals. The stability of the individual phages and the susceptibility of bacterial strains under various types of storage and for prolonged periods must also be studied.

SUMMARY AND CONCLUSIONS

The method used in the isolation, purification and characterization of bacteriophages for *Pseudomonas aeruginosa* has been presented. A set of 13 distinct phages was separated on the basis of host range, plaque morphology, mutation and cross resistance. These phages were employed in the classification of 161 strains of *Ps. aeruginosa* isolated from clinical sources in this hospital. It was possible to classify or type 88.8 per cent of these bacterial strains on the basis of the patterns of lysis produced by this set of typing phages. Some observations are presented on the relation of phage susceptibility to the reddish-brown color of some strains of *Ps. aeruginosa*. Studies on the significance of turbid plaque formation and its use in typing are also described.

The reproducibility of the phage patterns with any given strain and the similarity of the phage

pattern of different isolates from the same patient suggest that phage typing of *Ps. aeruginosa* may prove useful for epidemiological purposes.

ACKNOWLEDGMENTS

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A QUANTITATIVE STUDY OF RECYCLING OF ISOTOPE FROM GLYCINE–1–C^{14},α-N^{15} INTO VARIOUS SUBUNITS OF THE URIC ACID MOLECULE IN A NORMAL SUBJECT *

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Glycine is the precursor of three atoms of the purine ring, carbons 4 and 5 and nitrogen 7 (1, 2). When glycine-N^{15} is administered orally in man N^{15} is incorporated predominantly into N-7, but N^{15} is also found in N-1, N-3, and N-9 which are donated not by glycine but by aspartic acid (N-1) and the amide-N of glutamine (N-3 and N-9) (3). The N^{15} found in N-atoms other than N-7 of uric acid increases with time and may reach 35 per cent of the total N^{15} after several days (1). In contrast, when glycine-1-C^{14} is administered orally in man the isotope labels C-4 of uric acid almost exclusively, and very little C^{14} is incorporated into other carbon atoms by secondary pathways (4). As a consequence of these diverse fates of the α-N^{15} and 1-C^{14} atoms of glycine, the N^{15}/C^{14} ratio in uric acid exceeds 1.0 after administration of doubly labeled glycine (5). Values of 1.7 and 1.8 were reported in control subjects, and of 2.0 to 2.8 in gouty subjects by Gutman and co-workers (5). Their results further suggested that the ratio of (N^{15} in N-7)/(C^{14} in uric acid) was also greater than 1.0. In one "normal excretor" gouty subject the ratios were generally in excess of 1.2. Additionally, ratios of 1.08 to 1.92 (mean, 1.34) and of 0.86 to 1.37 (mean, 1.16) were calculated for two nongouty control subjects, the same (N^{15} in N-7)/(total N^{15} in uric acid) assumed as in one normal subject studied by Shemin and Rittenberg (1).

An incorporation of glycine-N^{15} into N-7 greater than of glycine-1-C^{14} into C-4 would have important implications that might signify either an accessory pathway of purine synthesis, or a disparity in the rates of "isotopic turnover" of the α-N and 1-C of glycine. There are known mechanisms whereby such a disparity of isotopic turnover might arise. A major fate of glycine involves interconversion with serine. The glycine-serine "shuttle" would not of itself alter the C^{14}/N^{15} ratio of reconstituted glycine, since it is the α-C of glycine that becomes the β-C of serine, and in the case of glycine-1-C^{14},α-N^{15}, the α-C is unlabeled. However, serine serves as a precursor of glyoxylic acid, a compound readily converted to glycine by glyoxylic-glutamic transaminase (6–8). The glyoxylic acid arising from glycine-1-C^{14},α-N^{15} via serine (9–12) would be unlabeled, since prior decarboxylation and deamination steps would have removed both C-1 and α-N of serine, and both carbons of glyoxylic acid would represent carbons originally in the α-position of glycine. Glycine can also be deaminated directly by glycine oxidase to form glyoxylic acid and ammonia (13). If this labeled glyoxylic acid was extensively diluted by unlabeled molecules, and if ammonia-N^{15} from glycine found its way rather directly into glutamic acid, it is theoretically possible that active resynthesis of glycine by the glyoxylic-glutamic transaminase route could result in reincorporation of N^{15} in excess of C^{14}. In this case the glycine pools should show more rapid isotopic turnover of C^{14} than of N^{15}, and such differential rates of turnover might be reflected in unequal labeling of various products synthesized from glycine. Such mechanisms might offer an explanation for the (N^{15} in N-7)/(C^{14} in uric acid) ratios more than 1.0 found by Gutman and colleagues in control subjects (5), and perturbations of certain reactions of these cycles might be present in gout to explain the higher ratios found in subjects with this disorder.

Since the calculation cited above, suggesting that relatively more N^{15} than C^{14} enters the glycine subunit of uric acid after injection of glycine N^{15}, 1-C^{14} in control subjects, also involved

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an *assumed* N^{15} partition in urate, it seemed desirable to explore this problem more fully. This study was performed to obtain direct evidence on incorporation of N^{15} into N-7 and of C^{14} into C-4 of uric acid after administration of glycine α-N^{15},1-C^{14} to a *normal* subject, and to determine how faithfully the glycine subunit of uric acid reflects the pattern of labeling of the glycine pool, as sampled during formation of hippuric acid. In this subject there was no evidence for disparate labeling of either the glycine subunit of uric acid or of glycine in hippuric acid. These results do not, of course, bear on the possibility of such disparity in subjects with derangements of purine or amino acid metabolism.

MATERIALS AND METHODS

Glycine-1-C^{14}, specific activity 4.0 mc per mmole, was purchased from Isotopes Specialties Co. Glycine-N^{15} was synthesized according to Schoenheimer and Ratner (14) from potassium phthalimide-N^{15}, containing approximately 95 atom per cent excess N^{15}, obtained from Isomet Corp.

Uric acid was isolated from urine by precipitation with copper (15) and repeatedly recrystallized from Li_2CO_3 solution (16), after decoloration with Darco-S51. All uric acid analyses were performed by the differential spectrophotometric method of Praetorius (17) employing purified uricase purchased from Worthington Biochemical Corp.

Hippuric acid was isolated according to Quick (18) and recrystallized repeatedly from hot water after decoloration with Darco until each sample gave an uncorrected melting point of 186° to 187° C.

Each uric acid sample was degraded by several procedures to permit analysis of individual atoms. C-(4 + 5) was obtained as glyoxylic acid semicarbazone according to Buchanan, Sonne and Delluva (19). N-7 was obtained as glycine by hydrolysis of uric acid in 12 N HCl at 160° for 18 hours (1). After alkalinization and aeration to collect NH_3 (20) derived from N-(1 + 3 + 9), the residual solution was neutralized and the glycine chromatographed on a Dowex 50-H^+ column, 1.0 × 7.5 cm. Any undegraded uric acid was washed off the column with water. Glycine was eluted with 0.1 M citrate, pH 3.4 (21), and was located with ninhydrin in the 30 to 70 ml fractions. Residual traces of NH_3 were retained by the column. N-(1 + 7) was obtained as NH_3 according to Brandenberger (22). Uric acid, and glycine derived from it, hippuric acid, and the glycine standard were all degraded by the micro-Kjeldahl procedure, and steam distilled to collect NH_3 for N^{15} analyses. Samples were prepared for N^{15} analysis according to Rittenberg (23). N^{15} analyses were performed in duplicate on a 180° Consolidated Electrodynamics mass spectrometer.[1] Samples checked on both instruments were in excellent agreement. All C^{14} determinations were performed on solid samples of uric acid, glyoxylic acid semicarbazone, glycine or hippuric acid in a Harvard-Robinson gas-flow counter (24). Most of these were done by plating samples in triplicate. All samples were corrected for self-absorption to a standard mass of 3.3 mg per 1.54 cm^2 planchet area. The efficiency of counting at this sample thickness is 24.6 per cent.

Design of experiment. The subject of the study was a 27 year old normal adult male weighing 84.0 kg. He was placed on a standard low purine diet containing 2,600 calories, 70 g protein and 350 g carbohydrates, which was prepared by the diet kitchen of Duke Hospital and which he ingested throughout the study. After 5 days of pretreatment with this diet, the subject's serum uric acid was 5.4 mg per 100 ml and the 24-hour urinary uric acid was 523 mg. These values varied only minimally throughout the next 24 days, the urinary uric acids ranging from 452 to 529 mg per day, averaging 495 ± 18 mg per day. Urinary uric acid and hippuric acid isolated before labeled glycine was given showed no biological enrichment with N^{15}. The subject received 5.01 g C^{14}, N^{15} glycine orally. This glycine showed by analysis 95.1 atom per cent excess N^{15}, and 886,340 cpm per mmole (mol wt of glycine, corrected for N^{15} content, 76.02). The total N^{15} administered was 293.0 mg; total C^{14} was 57,533,000 cpm.

Urine samples were collected in sequential aliquots of 10, 2, 10, and 2 hours, respectively, during the first 2 days. Thereafter they were collected in sequential 22- and 2-hour aliquots for 22 additional days. Uric acid was isolated from the 10- and 22-hour urine samples. Hippuric acid was isolated from urine samples collected for 2 hours after intravenous administration of 400 mg sodium benzoate. All samples were analyzed in triplicate for uric acid content. The concentrations of N^{15} and C^{14} in uric acid of these 2-hour samples were obtained by interpolation from the plots of data obtained from analysis of uric acid isolated from 10- or 22-hour samples.

RESULTS

Gross incorporations of N^{15} and C^{14} into uric acid. The incorporations of N^{15} and C^{14} into individual samples of uric acid are shown in Figure 1. Both incorporation curves show secondary maxima on Days 10 to 12 in addition to the early maxima on Days 2 and 3. As anticipated, the incorporation of N^{15} exceeded that of C^{14}, and the disparity increased gradually with time. By the end of the 24-day period, an N^{15}/C^{14} ratio of 1.5 had been reached. The cumulative incorporation of N^{15} into

[1] We are indebted to Mr. William Comstock and Dr. DeWitt Stetten, Jr., of the National Institutes of Health, for the majority of N^{15} analyses, and to Dr. Henry Kamin and Mr. Bernard Bulos of the Department of Biochemistry of Duke University Medical Center for some of the total uric acid-N^{15} and hippurate-N^{15} analyses.

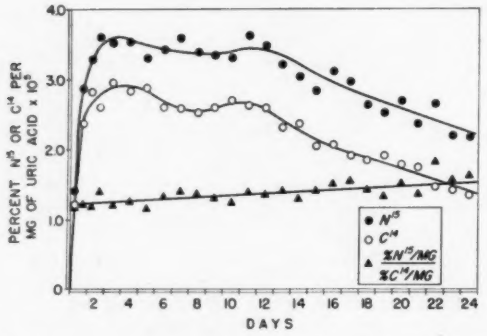

FIG. 1. INCORPORATION OF N^{15} AND C^{14} INTO URIC ACID AFTER ORAL ADMINISTRATION OF GLYCINE-α-N^{15},1-C^{14}.

uric acid was 0.360 per cent in 24 days, whereas that of 1-C^{14} was 0.264 per cent during the same period.

Incorporation of N^{15} into individual nitrogen atoms of uric acid. Values for incorporation of N^{15} into N-1 and N-(3 + 9), in atom per cent excess, may be calculated from analytical data as follows.

$$N^{15}\text{-}1 = 2\left[\frac{N^{15}\text{-}(1+7)}{2}\right] - (N^{15}\text{-}7)$$

$$N^{15}\text{-}(3+9) = 3\left[\frac{N^{15}\text{-}(1+3+9)}{3}\right] - (N^{15}\text{-}1)$$

Since both N-3 and N-9 are derived from the amide-N of glutamine (3), the enrichment of each may be taken as approximately ½ [N^{15}-(3 + 9)], assumed that the same pool of intracellular glutamine contributes both N-3 and N-9, and that the turnover of this pool is slow with respect to the time lapse between these contributions. Thus, individual enrichment values may be obtained for N-1, N-3, and N-9 in addition to N-7. The cumulative incorporations of N^{15} into individual N-atoms are shown in Figure 2, in which the sum of N^{15} in N-1, N-3, N-7, and N-9 is also shown. The sum of the individual N^{15} values is in excellent agreement with values of uric acid itself. In no case did the values on any given day differ by more than 10 per cent. The cumulative incorporation of N^{15} into uric acid, as determined by addition of the cumulative incorporation values into the individual N-atoms (Figure 2) was 0.260 per cent. This value is virtually identical with that obtained by analysis of intact uric acid samples.

N^{15} in N-7 of uric acid accounted for 88 per

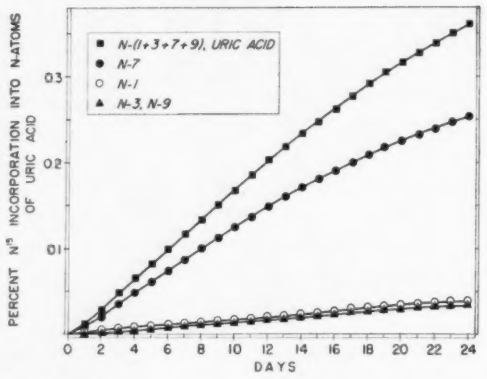

FIG. 2. INCORPORATION OF N^{15} INTO INDIVIDUAL N-ATOMS OF URIC ACID. The triangles represent N^{15} in N-3 and N-9 individually; they are assumed to be equally labeled (see text). The squares represent the sums of the individual values, and also the N^{15} in total uric acid, since these are virtually identical (see text).

cent of the N^{15} in uric acid collected over the first 10 hours. By the end of the third week, however, it accounted for only about 60 to 65 per cent of the total N^{15} (Figure 3). These data show a general conformity with those of Shemin and Rittenberg (1).

Incorporation of C^{14} into C-4 of uric acid. When uric acid was degraded to glyoxylic acid semicarbazone, the enrichment of the latter in all cases approximated that of uric acid itself. The data are given in Table I. The average ratio of enrichment of glyoxylic acid semicarbazone to uric acid was 0.981, signifying that virtually all of the C^{14} of the uric acid molecule was present in

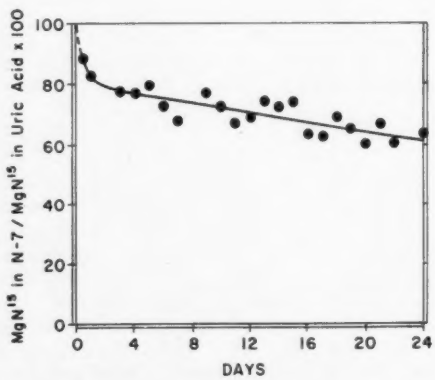

FIG. 3. PERCENTAGE OF N^{15} FOUND IN THE N-7 ATOM OF URIC ACID VERSUS TIME.

TABLE I

Comparison of total enrichment of uric acid and of the C-(4 + 5) fragment of uric acid, isolated as glyoxylic acid semicarbazone

Day	C-(4+5)	Uric acid	C-(4+5)/ uric acid
	cpm/mmole	*cpm/mmole*	
3	2,738	2,859	0.96
4	2,659	2,758	0.97
5	2,646	2,804	0.94
6	2,702	2,506	1.08
7	2,568	2,503	1.03
8	2,528	2,412	1.05
9	2,371	2,489	0.95
10	2,621	2,608	1.00
11	2,337	2,535	0.92
12	2,332	2,510	0.93
14	2,333	2,302	1.01
15	1,977	1,962	1.01
16	1,978	1,993	0.99
17	1,821	1,853	0.98
18	1,716	1,784	0.98
19	1,702	1,844	0.92
20	1,637	1,717	0.95
21	1,565	1,704	0.92
22	1,454	1,401	1.04
		Average	0.981

the C-(4 + 5) fragment. Although in this study the C-6 and C-(2 + 8) fragments of uric acid were not isolated, in an earlier study of eight samples from four subjects any isotope that may have been in these fragments after administration of glycine-1-C^{14} was too low in concentration to be detected when counted as $BaCO_3$ (4).

In these earlier studies of the distribution of C^{14} in the various carbon atoms of urinary uric acid, from 95 to 100 per cent of the isotope was found in the C-(4 + 5) fragment. Two samples of glyoxylic acid semicarbazone derived from C-(4 + 5) were further degraded. Only a very small amount of activity (< 10 per cent) was detected in CO_2 derived chiefly from C-5, and since the degradation method employed (4, 19) is known to separate C-4 and C-5 imperfectly, this was considered contamination from C-4. This explanation may be applied also to the small degree of apparent labeling of C-5 found by Buchanan and colleagues (19) and by Karlsson and Barker (2) after experiments with glycine-1-C^{14} in pigeon systems. Significant labeling of C-5 occurs only under circumstances in which glycine 2-C^{14} is employed (2) or in which the "formyl pool" itself becames highly labeled and contributes isotope to C-(2 + 8) and by indirect pathways to C-5 also (25). Secondary enrichment of C-5, from glycine-1-C^{14}, is most unlikely, since even administration of $C^{14}O_2$ or formate-C^{14} does not result in enrichment at this position beyond 1 to 3 per cent of the total label in uric acid (2, 26). In the present study there is no significant fraction of C^{14} unaccounted for that could be in C-(2 +8) or C-6. Therefore it appears reasonable to conclude that virtually all C^{14} is in C-4 of uric acid after administration of glycine-1-C^{14} although, admittedly, rigorous proof is lacking.

Comparison of N^{15} in N-7 and of C^{14} in C-4. The isotopic enrichments of N-7 and C-4 are plotted in Figure 4, where it can be seen that there is no significant difference in the concentrations of N^{15} and C^{14} recovered in the glycine subunit of uric acid. Comparison of 19 individual samples on which both analyses were available gave an N^{15}/C^{14} incorporation ratio of 1.009. Individual values ranged from 0.865 (Day 2) to 1.118 (Day 9). The secondary maxima previously observed in the gross incorporation data (Figure 1) are again in evidence in N-7 and C-4. These maxima in uric acid on Days 10 to 12 may reflect a contribution of granulocyte nucleic acid purines, since granulocytes have a life span of about 10 days (27).

The cumulative incorporations of N^{15} into N-7 and of 1-C^{14} into C-4 are shown in Figure 5. Again, good agreement is seen. The values differ by only 0.005 per cent after 24 days.

Analysis of urinary hippuric acid. Small doses of sodium benzoate (400 mg) were given periodically to promote synthesis of hippuric acid. Quantities of 1.5 to 3.0 g of benzoate have been used by others (28, 29), but under these circumstances

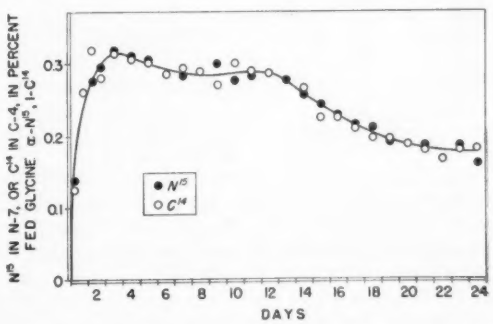

FIG. 4. INCORPORATION OF N^{15} INTO N-7 AND OF C^{14} INTO C-4 OF URIC ACID, AFTER ORAL ADMINISTRATION OF GLYCINE-α-N^{15},1-C^{14}.

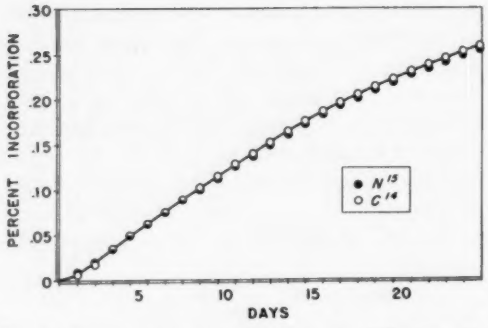

FIG. 5. CUMULATIVE INCORPORATION OF N^{15} INTO N-7 AND OF C^{14} INTO C-4.

benzoate stimulates mobilization of glycine from protein or synthesis of new glycine (28, 30), giving lower enrichment values of hippurate. The smaller dose was chosen to minimize these phenomena. The hepatic glycine pool concerned with synthesis of hippurate (31) was sampled to permit comparison of its N^{15}/C^{14} concentrations with those of administered glycine as functions of time. The values are plotted on semilogarithmic coordinates in Figure 6, where it is apparent that there is no significant differential in turnover of one isotope in glycine with respect to the other in the pool participating in hippuric acid synthesis. Although the analytical data on individual samples of hippuric acid are somewhat more erratic than on the glycine subunit [C-(4 + 5) and N-7] of uric acid, the N^{15}/C^{14} ratio on 19 samples of hippuric acid on which both analyses were obtained averaged 1.002.

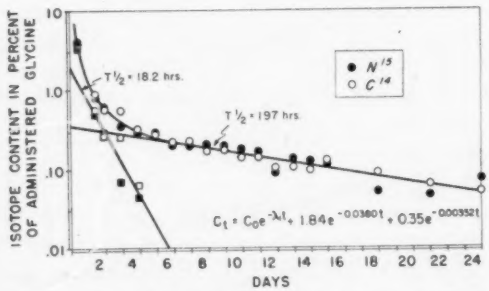

FIG. 6. SEMILOGARITHMIC PLOT OF N^{15} AND C^{14} CONTENT OF HIPPURIC ACID VERSUS TIME. The squares represent the differences between the values of N^{15} and C^{14} observed and those anticipated from the regression line, $T_{\frac{1}{2}} = 197$ hours, and these data (■ = N^{15}, □ = C^{14}) serve as the basis for placement of the regression line, $T_{\frac{1}{2}} = 18.2$ hours. The difference points on Day 1 fall well above the second line and signify the existence of a fractional turnover of the glycine pool too rapid to be analyzed from available data (see text).

The semilogarithmic plot of isotope decay in hippuric acid indicates at least three fractional turnover rates of the glycine pool. The data fit the equation

$$C_t = C_0 e^{-\lambda_1 t} + 1.84e^{-0.0380t} + 0.35e^{-0.00352t}$$

The value $\lambda_2 = 0.0380$ is in good agreement with the second rate constant calculated by Watts and Crawhall, $\lambda_2 = 0.0377$, after administration of glycine-1-C^{13} to a normal subject (32). They also found a more rapid component of glycine turnover with a rate constant of $\lambda_1 = 0.552$; in the present study no samples were obtained earlier than 10 hours after glycine administration, and data are therefore insufficient to plot the most rapid component of glycine turnover. The rate constants calculated are somewhat greater than those found by Berlin, Hewitt and Lotz (29) in three subjects given glycine-2-C^{14}, but in their subjects some recycling of C^{14} from the 2-position back into resynthesized glycine was evident.

DISCUSSION

This study demonstrates that in this normal subject all of the incorporation of N^{15} into N-7 of uric acid is accounted for on the basis of incorporation of the intact glycine molecule. The processes that result in transfer of N^{15} from glycine to aspartic acid and the amide-N of glutamine, and secondarily into N-1, and N-3 and N-9 of uric acid, respectively, do not result in detectable relabeling of glycine. Had this occurred, N^{15}/C^{14} values significantly in excess of 1.0 would have been expected in N-7/C-4 of uric acid, and in hippuric acid.

Recycling of N^{15} from glycine into the "nitrogen pool" and back into endogenously produced glycine remains a possible explanation for [(N^{15} in N-7)/(C^{14} in uric acid)] > 1.0 found in gouty subjects by Gutman and colleagues (5). The pathways by which $N^{15}H_4^+$ is incorporated excessively in such patients into uric acid might well include labeling of N-7 via glycine. A detailed analysis of these possibilities in gouty patients is warranted.

The present study shows, however, that in this normal subject there is no disparity of isotopic

turnover of α-N^{15} and 1-C^{14} in body glycine, and no evidence for other than direct incorporation of intact glycine into the glycine subunit of uric acid.

SUMMARY

1. Glycine-1-C^{14},α-N^{15} was administered orally to a control subject, and incorporations of C^{14} and N^{15} into individual atoms of uric acid, and into hippuric acid, were followed for 24 days.

2. Glycine-1-C^{14} labeled C-4 of uric acid almost exclusively. Glycine N^{15} labeled N-1, N-3, and N-9 appreciably, in addition to its direct incorporation into N-7. The percentage of N^{15} in atoms other than N-7 reached 35 to 40 per cent in 3 weeks.

3. A parallel incorporation of N^{15} and C^{14} into N-7 and C-4 of uric acid was observed. Analyses of hippuric acid similarly gave no evidence for differential rates of isotopic turnover of α-N and carboxy-C of glycine. The data indicate that all of the N^{15} in N-7 of uric acid in this normal subject was accounted for by direct incorporation of the intact glycine molecule; there was no detectable recycling of N^{15} into N-7 by secondary pathways.

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CALCIUM METABOLISM OF BONE *IN VITRO*. INFLUENCE OF BONE CELLULAR METABOLISM AND PARATHYROID HORMONE*

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As has been pointed out by McLean and Urist (1) and by Neuman and Neuman (2), the total Ca concentration in the circulating fluids can be considered to consist of two parts—that which is the result of the passive solubility of bone mineral, and that which is the result of bone cellular metabolic phenomena. These metabolic phenomena are thought to raise the Ca concentration above the level predicted by bone salt solubility and to be influenced by parathyroid hormone.

By analogy, in an *in vitro* system the final concentration of Ca (and P) in the medium surrounding living bone samples should be partly the result of passive solubility equilibrium between bone mineral and the medium and partly the result of cellular metabolic activity. It has been shown in previous communications from this and other laboratories (3–5) that living samples of bone survive *in vitro* for appreciable periods and carry on active metabolism which can be modified by various changes in conditions. Measurement of Ca and P concentrations under steady state conditions in the incubation medium of such an *in vitro* system appeared to offer an opportunity to explore the influence of various aspects of cell metabolism upon the mobilization of these ions from bone. In addition, by incubating bone samples in which cellular activity had been destroyed, the fraction of the total concentration that could be assigned to passive solubility phenomena could be estimated.

The studies reported below were undertaken: first, to determine which fraction of the steady state Ca and P concentrations in the media could be attributed to passive solubility of bone mineral and which fraction appeared to depend upon the metabolic activity of cells; second, to examine more directly which steps in the metabolic sequence and which metabolites of cells might be associated with mobilization of mineral from bone; and finally, to explore the effects prior treatment of the animals with parathyroid hormone might have on these phenomena.

The results of these experiments indicate that 18 per cent of the Ca concentration maintained by normal living bones in their surrounding media was related to metabolic phenomena, about one-half of this being associated with glucose metabolism. No direct correlation, however, could be found between rates of lactate production (previously shown to be the chief end-product of glucose metabolism in this tissue) and Ca concentration. Parathyroid hormone treatment increased the total concentration of calcium 17 per cent above normal controls. The mechanism of this action appeared to involve both an increase in the passive solubility of the bone mineral and changes in the pattern of cellular metabolic effects. No effects on total P concentrations were observed in any of the experiments with surviving bone samples.

METHODS

A. Preparation of tissue samples. Adult white Swiss mice, 7 to 9 weeks old, were killed by cervical vertebra fracture, and the calvaria removed. The bones were freed from tendinous and muscular attachments by dissection, and adherent blood and clots were removed by washing the samples in ice-cold Krebs-Ringer bicarbonate medium. All bones were kept in fresh media stored on ice until the start of the incubation. After gentle blotting, two calvaria were pooled (about 100 mg total weight), weighed, and placed in the incubating flask.

B. Heat-inactivation. When bone samples were killed by heating, the procedure was as follows. Samples of glucose-free medium were heated to boiling, removed from the gas flame, and the bone samples quickly added and kept in the hot media for 1.5 minutes. They were then transferred to freshly prepared media for incubation.

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† Markle Scholar in Medical Science.

C. Incubation. Unless otherwise indicated the samples were incubated in a Dubnoff metabolic incubator at 37.8° C in 2 ml of modified Krebs-Ringer media,[1] buffered to pH 7.4 with HCO_3^- and 5 per cent CO_2. Penicillin at a final concentration of 5 U per ml and streptomycin, 0.01 mg per ml, were added to prevent bacterial growth. Glucose was used as substrate at a concentration of 2 mg per ml. During the incubation a continuous stream of 95 per cent O_2 with 5 per cent CO_2 was fed into the hood covering the flasks, which were left in the incubator without stoppers. All media were also equilibrated for 10 minutes with the appropriate gas before being placed in the flasks. Iodoacetate, when used, was at a final concentration of 0.9×10^{-3} M (6). Normal controls were incubated and compared with the experimental samples for each experiment.

D. Injection of parathyroid extract[2] *(PTE).* Hormone-treated mice were injected subcutaneously with 0.15 ml of PTE once daily for 3 successive days, the last injection being given 18 hours prior to sacrifice. The control group was injected with 0.15 ml of distilled water at the same time.

E. Analytical techniques. Aliquots of the media were analyzed for Ca by the EDTA-calcium chelating method with murexide as an indicator,[3] for P by the method of Fiske and Subbarow (7), and for lactate by a modification of Barker and Summerson's technique (8); pH in the media was determined by glass electrode with a Beckman pH meter, model G. In the experiments in which pH was measured, the flasks were gassed separately through rubber stoppers. The pH was measured exactly 1.5 minutes after the stopper had been removed from the flask in order to equalize losses of CO_2 from the media that would affect pH.

F. Calculation. There was a tendency for minor shifts to occur in the absolute values from one day to another, while the relative differences between controls and experimental samples remained quite constant. For example, on one day the mean Ca value (4 incubations) was 0.64 mmole per L for viable samples and 0.52 (3 incubations) for heat-inactivated samples. On another day the mean values (3 incubations in each case) obtained were 0.58 and 0.47. The differences were 0.12 and 0.11 in these two experiments and in each case were significantly different, with a p value below 0.001. Comparable differences in phosphate concentrations from day to day were observed. Therefore, all values have been calculated as per cent of normal controls for each experiment. In all experiments values obtained with living bone samples incubated in standard media with glucose under aerobic conditions have been used as normal controls. Bessel's correction for small samples was used in calculating standard deviations. The significance of the differences between means was estimated by means of Students' *t*-test.

[1] The media contained no Ca; P was present at a concentration of 0.40 mmole per L.

[2] Kindly supplied by Dr. Otto K. Behrens, Eli Lilly Co.

[3] O. A. Iseri and P. L. Munson, personal communication.

G. Criterion of bone cell viability. In experiments where impairment of the cells might have been expected as a result of the incubating conditions, it was important that the viability of the bone samples be determined. Since lactate production is a prominent feature of the metabolism of bone cells *in vitro* (3), the rate of lactate production compared with controls in a subsequent incubation under normal conditions was used to judge viability in living samples. On the other hand, when bone samples were inactivated by heat, it was equally important to be sure that the heating procedure resulted in complete and permanent cessation of cellular metabolism. Inability of these samples to produce lactate was taken as evidence that their cells were dead.

RESULTS

A. Time required to reach steady state and determination of passive solubility component of Ca and P concentrations

A significant period of incubation was required before the steady state concentrations of Ca and P, which the sample would maintain in the medium, were reached. Since a steady state concentration was to be used as the end-point in these studies, the determination of an appropriate time of incubation was important. It was equally important that the concentrations to be expected on a passive solubility basis be defined, if the role of cellular metabolism was to be examined. Therefore, Ca and P concentrations in media were

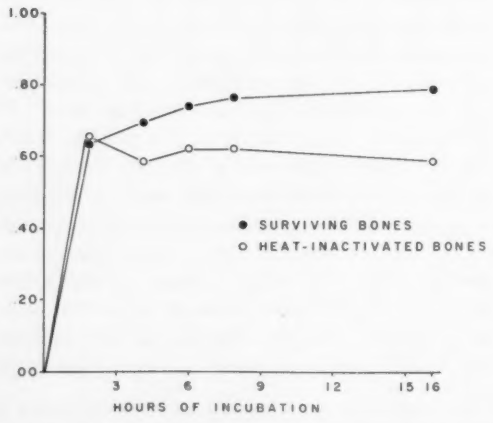

FIG. 1. MEAN CA CONCENTRATIONS IN THE MEDIA SURROUNDING SURVIVING AND HEAT-INACTIVATED BONES. Data at 6 hours are the same as shown in Table I. Each point represents the mean of at least 8 incubations. Values on the ordinate are mmoles per L.

TABLE I

Effects of heat inactivation on bones from normal and PTE-treated animals

Exp. day	Incub., hours	Ca			P			Ca × P		
		Controls, normal	Heated, normal	Heated, PTE-R	Controls, normal	Heated, normal	Heated, PTE-R	Controls, normal	Heated, normal	Heated, PTE-R
1	6	0.64 (4)*	0.52 (3)	0.63 (4)	1.11 (4)	1.03 (3)	0.98 (4)	0.71 (4)	0.54 (3)	0.61 (4)
2	6	0.58 (3)	0.47 (3)	0.56 (2)	0.83 (3)	0.82 (3)	0.80 (2)	0.48 (3)	0.39 (3)	0.45 (2)
3	6	0.59 (3)	0.50 (3)	0.59 (3)	0.77 (3)	0.72 (3)	0.73 (3)	0.46 (3)	0.35 (3)	0.44 (3)

* Figures in parentheses indicate in all tables the number of experiments in each group.

measured after different periods of incubation of living and heat-inactivated samples. The data obtained in these experiments are shown in Figure 1 and Tables I and II.

The data plotted in Figure 1 indicate that a steady state for Ca concentration in the medium surrounding heat-inactivated bone samples was established by 2 hours, while the maximal concentration for living samples was not reached until 16 hours, although it was closely approached by 6 hours. Practically identical curves were obtained for P concentration.

These results (Figure 1) suggested that the greatest differences in Ca concentration between living and dead bone samples would be observed if the incubations were conducted for 16 hours, but, as can be seen from Table II, bone samples incubated for this period of time without glucose were no longer viable. This indicated that if the relation of glucose metabolism to Ca and P concentrations in the medium was to be examined, an incubation period shorter than 16 hours would be required. Six hours was selected as a suitable time. In addition, incubation for 4 hours was conducted in some experiments.

The concentrations of calcium observed for living samples after 6 hours varied from day to day between 0.58 and 0.78 mmole per L (mean, 0.76) while inorganic phosphorus concentrations varied between 0.72 and 1.11 mmoles per L (mean, 0.92). These mean values are both below those expected in the extracellular fluid of this species on the basis of serum analysis, although the relatively higher phosphate concentration is present in the serum also. The very slowly rising values (shown for Ca in Figure 1) with increasing periods of incubation beyond 6 hours suggest that, had it been possible for the incubation time to be much longer, values more like extracellular fluid might have been reached. Unfortunately this was not practicable, since cellular metabolism becomes very variable and tends to slow down or cease when incubations of more than 24 hours are carried out.

TABLE II

Effects of glucose deprivation; normal bone

Exp. day	Incub., hours	Ca	
		+Glucose	− Glucose
4	16	0.73 (3)	0.66 (3)
5	16	0.60 (6)	0.52 (6)
6	16	0.72 (3)	0.66 (3)
7	16	0.80 (3)	0.72 (3)
Viability of samples		+	0

Ca, P, and Ca × P concentrations found in media after 6-hour incubations of living and heat-inactivated samples are shown in Table I. Since Ca, P, and Ca × P concentrations were lower for heat-inactivated samples, it can be inferred that 82.4 per cent of the Ca concentration, 95.2 per cent of the P concentration, and 78.1 per cent of the Ca × P concentration were the result of passive mineral solubilty. The balance could be attributed to cellular activity. The depression in Ca concentration produced by inactivation of cells due to prolonged deprivation of glucose (Table II) approached that produced by heat-inactivation. This finding, together with the similarity in Ca concentration at 2 hours between living and heat-inactivated samples (Figure 1) suggests that in both experiments the reduction was the result of cessation of celluiar metabolism rather than of changes in mineral solubility produced by heat or changes in Ca and P diffusion produced by the heating process.[4]

[4] Additional evidence in favor of this view was obtained from incubations at a low pH where no differences were seen between living and heat-inactivated bones (unpublished studies).

TABLE III

Effects of glucose deprivation; normal bone

Exp. day	Incub., hours	Ca		P		Ca × P		(Glucose-ō glucose)	
		+Glucose	ō Glucose	+Glucose	ō Glucose	+Glucose	ō Glucose	Ca	P
8	4	0.70 (4)	0.63 (4)	0.62 (4)	0.60 (4)	0.45 (4)	0.39 (4)	+0.07	+0.02
9	4	0.78 (4)	0.70 (4)	0.64 (4)	0.61 (4)	0.50 (4)	0.43 (4)	+0.08	+0.03
10	4	0.77 (4)	0.73 (4)					+0.04	
11	4	0.91 (4)	0.85 (3)	0.55 (4)	0.59 (4)	0.50 (3)	0.50 (3)	+0.06	−0.04
12	4	0.63 (4)	0.61 (4)	0.59 (4)	0.62 (4)	0.37 (4)	0.38 (4)	+0.02	−0.03
13	4	0.80 (4)	0.73 (4)	0.56 (4)	0.58 (4)	0.45 (4)	0.43 (3)	+0.07	−0.02
14	6	0.78 (3)	0.68 (3)	0.99 (3)	1.01 (3)	0.78 (3)	0.69 (3)	+0.10	−0.03
15	6	0.71 (3)	0.66 (3)	0.93 (3)	0.96 (3)	0.66 (3)	0.64 (3)	+0.05	−0.03
16	6	0.74 (2)	0.65 (2)	0.95 (2)	0.90 (2)	0.71 (2)	0.59 (2)	+0.09	+0.05
17	6	0.66 (3)	0.64 (3)	1.07 (3)	1.04 (3)	0.71 (3)	0.67 (3)	+0.02	+0.03

B. Effects of changes in glucose metabolism

1. Glucose deprivation. While glucose deprivation for 16 hours was not compatible with survival, 4 and 6 hours without this substrate was well tolerated since, in a subsequent incubation of the same samples with glucose, the lactate production was resumed at close to the normal rate. As can be seen from Table III, lack of glucose depressed Ca concentration 7 and 8.2 per cent and lactate production 91.1 and 94.1 per cent after 4 and 6 hours, respectively. The Ca × P products were similarly reduced. In none of these experiments was there any difference in P concentrations, while the differences in Ca concentrations, although small, were highly significant. Differences in the Ca × P product (due almost wholly to changes in Ca) were statistically significant after 6 hours.

In separate experiments the influence of the presence of glucose on heat-inactivated bones was studied. No significant differences in Ca or P between samples incubated with and without glucose were found and, hence, the possibility was excluded that the effects of glucose were unrelated to its metabolism.

2. Iodoacetate inhibition. In an attempt to localize the effect of glucose metabolism, glycolysis was blocked at the triose phosphate dehydrogenase level with iodoacetate (IA). When this inhibitor was added to the media (Table IV), Ca was depressed 5.1 and 5.9 per cent and lactate 94.1 and 94.5 per cent after 4 and 6 hours. The Ca × P product was decreased 7.9 per cent at 6 hours. Again the differences in Ca were highly significant, while P was not significantly influenced. However, two possibilities exist that this effect of IA was not due solely to interference with glucose metabolism. First, the IA ion as such might have an effect on the passive solubility of bone mineral, and second, IA might kill the cells (9); but, when samples were incubated without substrate, or with pyruvate instead of glucose, IA had no significant influence on Ca concentrations. If the cells had been killed, lower Ca concentrations would have been expected on the basis of the data obtained with heat or glucose deprivation, as shown in Tables I and II. On the other hand, if IA had had

TABLE IV

Effects of iodoacetate (IA) inhibition; normal bone

Exp. day	Incub., hours	Ca		P		Ca × P	
		Controls, normal	+IA, normal	Controls, normal	+IA, normal	Controls, normal	+IA, normal
18	4	0.66 (4)	0.62 (4)				
19	4	0.80 (3)	0.75 (3)				
20	4	0.69 (3)	0.66 (3)				
21	6	0.71 (6)	0.67 (6)	0.89 (6)	0.86 (6)	0.64 (6)	0.58 (6)
22	6	0.70 (4)	0.65 (4)	0.87 (4)	0.88 (4)	0.61 (4)	0.58 (4)

TABLE V

Effects of anaerobiasis; normal bone

Exp. day	Incub., hours	Ca		P		Ca × P	
		Controls, normal	Anaerobic, normal	Controls, normal	Anaerobic, normal	Controls, normal	Anaerobic, normal
23	4	0.78 (4)	0.76 (4)	0.64 (4)	0.62 (4)	0.50 (4)	0.47 (4)
24	4	0.80 (4)	0.80 (4)	0.63 (4)	0.64 (4)	0.50 (4)	0.51 (4)
25	4	0.80 (4)	0.75 (4)	0.64 (4)	0.62 (4)	0.51 (4)	0.47 (4)
26	6	0.75 (6)	0.75 (6)	0.91 (6)	0.93 (6)	0.68 (6)	0.71 (6)
27	6	0.70 (4)	0.66 (3)	0.87 (4)	0.90 (3)	0.61 (4)	0.60 (3)

a direct effect on passive mineral solubility, this should have been reflected by change in Ca concentration in experiments using samples incubated without substrate or with pyruvate.

3. Anaerobiasis. It has been suggested by other workers that the metabolically dependent fraction of the circulation Ca^{++} concentration might be due to the local production of citric acid by the bone cells (2, 10–14). However, the amounts of citrate produced by bone cells *in vitro* is extremely small (1 to 2 per cent) compared with lactate (3). Furthermore, since about 80 per cent of the glucose utilized by the cells could be accounted for as lactate, it was suggested that lactic acid might be of primary and citric acid of only secondary importance in mobilizing Ca and P from bone mineral *in vivo* (3). For these reasons it was of interest to explore the effects of increased rates of lactate production on Ca and P concentrations.

In Table V the results of experiments in which samples were incubated anaerobically (nitrogen 95, CO_2 5 per cent) are compared with controls under aerobic conditions. The results are basically the same after 4 and 6 hours. Anaerobically, 15.5 to 17.8 per cent more lactate was produced, while the Ca, P, and the Ca × P products were not significantly affected. In fact, in both series of experiments Ca values, the chief variable, were slightly depressed. These experiments therefore suggest that the rate of lactate production per se is not the critical factor involved in the mobilization of Ca from bone mineral.

C. Studies with bone from animals treated with parathyroid hormone

Bones from mice injected with parathyroid hormone were incubated for 6 hours with and without glucose and compared with controls. Thus, each experiment consisted of four groups. There were three samples in each group, and four such experiments were conducted. The values obtained are shown in Table VI and Figure 2. Three points of interest should be noted.

First, in the hormone-treated group with glucose present as substrate the concentrations of Ca and the Ca × P product were higher than for their normal counterparts. This is in agreement with the findings of others (15, 16). Second, the glucose effects on the concentrations of Ca and the Ca × P product were relatively more pronounced in the hormone-treated group than in controls, suggesting synergistic effects of glucose and hormone on the mobilization of Ca. The relative differences between samples with and without glucose for both groups are shown in Figure 2. This pattern was the same in all ex-

TABLE VI

*Effects of treatment with PTE (in vivo) and of glucose**

Exp. day	Incub., hours	Ca		P		Ca × P		(Glucose-s̄ glucose)	
		+Glucose	s̄ Glucose	+Glucose	s̄ Glucose	+Glucose	s̄ Glucose	Ca	P
14	6	0.92 (3)	0.74 (3)	1.00 (3)	0.96 (3)	0.92 (3)	0.71 (3)	+0.18	+0.04
15	6	0.83 (3)	0.76 (3)	0.92 (3)	0.88 (3)	0.77 (3)	0.67 (3)	+0.07	+0.04
16	6	0.84 (2)	0.70 (2)	0.88 (2)	0.85 (2)	0.73 (2)	0.60 (2)	+0.14	+0.03
17	6	0.77 (3)	0.68 (3)	1.03 (3)	1.04 (3)	0.80 (3)	0.71 (3)	+0.09	−0.01

* The data for samples from normal animals incubated on the same day appear in Table III.

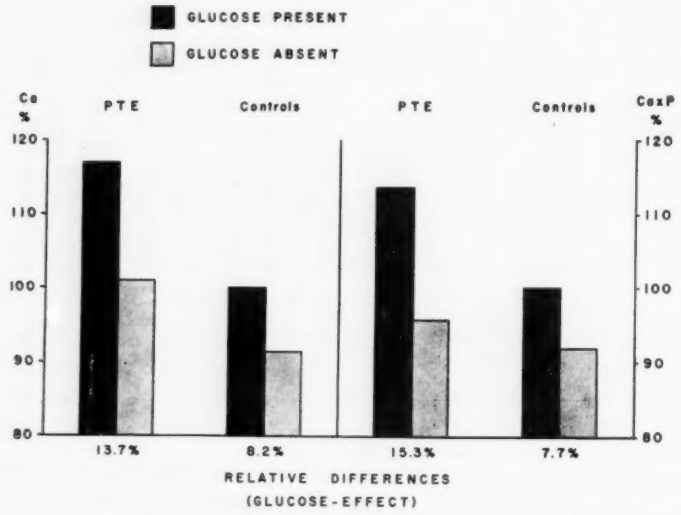

FIG. 2. "STEADY STATE" CA AND CA × P CONCENTRATIONS IN THE MEDIA SURROUNDING BONES FROM NORMAL AND PTE-TREATED ANIMALS. Data from Table VI. Note the increase in the relative differences between samples incubated with and without glucose in the PTE group.

periments reported here and in a preliminary experiment run for 4 hours. There are several reports showing that the hormone is capable of modifying cell metabolism (4, 15–17), and in the present study such effects were demonstrated by an increase in lactate production of 22.3 per cent. Third, it appears that the values for Ca concentration and the Ca × P product were higher for the hormone-treated samples than for controls even without glucose, although the difference for the product was not significant. These findings suggested that the passive solubility of the bone mineral might have been increased by parathyroid treatment and that part of the increases in Ca concentration might have been the result of increases in passive solubilization of the mineral.

This suggestion was supported by the results of subsequent experiments using heat-inactivated bones from PTE-treated animals. The Ca concentrations and the Ca × P concentration products were significantly higher in these samples than in untreated heat-inactivated samples incubated at the same time (Table I).[5] The Ca concentrations observed were practically as high as for normal, living bones supplied with glucose. Thus, the mineral of bones from PTE-treated animals appears to have an increased solubility independent of metabolism of surviving cells.

D. Effects of final pH in the medium

It is well known that the solubility of bone mineral increases as pH decreases. Thus, if in

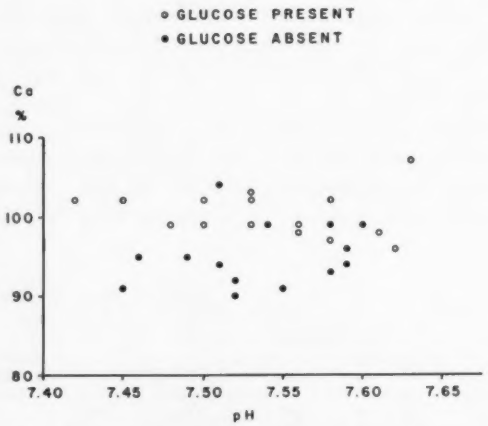

FIG. 3. "STEADY STATE" CA CONCENTRATIONS VS FINAL PH IN THE MEDIA SURROUNDING BONES INCUBATED WITH AND WITHOUT GLUCOSE. Note lack of correlation between Ca concentrations and final pH.

[5] This was true even after the bones had been washed by incubation for 1 hour before the ordinary incubation in fresh media.

these systems major pH changes occurred *in the media* during incubation, such changes alone might have been responsible for the mobilization of Ca from the bone samples. However, pH remained quite constant in the media including those in which pH changes might have been possible due to acid production by actively metabolizing cells. The variations that did occur could not be correlated with the final Ca concentrations observed. In Figure 3, pH values at the end of incubation are plotted against Ca concentrations to illustrate this point. The values are from experiments in which the effects of the presence of glucose were studied. It is apparent that only minor pH variations occurred, and these were practically equally distributed between the two groups. Also, Ca concentrations were independent of pH in this range. In the other experiments of this series a similar lack of correlation between Ca concentration and final pH of the medium was found.

DISCUSSION

In this study a correlation has been demonstrated between the rate of bone cellular metabolism and steady state Ca concentrations in the media. Except for the experiments with heat-inactivated bones, no significant changes were obtained for P concentrations. When changes were seen in the Ca × P product, they were due to changes in Ca. Just why this was true is not clear, but in view of the fact that bone mineral may exhibit molar Ca/P ratios from 1.3 to 2, differences in Ca may be more pronounced than for P when bone salt goes into solution. Furthermore, in equilibration studies with human powdered bone it has been demonstrated that lowering pH from about 7.8 to 6.6 resulted in steadily increased Ca concentrations, while total P concentrations remained unchanged (18). Consequently, if the metabolic effects demonstrated in the present studies were related to acid production and lowering of pH *at the crystal surface* (rather than in the medium), no change in total P might be seen. It should be noted that were citric acid the important metabolite, changes in P would be expected from Neuman's data, since he and his collaborator have shown that the increased solubility of hydroxyapatite caused by citrate in the surrounding fluid is accompanied by increases in phosphate which appears to be displaced from the crystals by citrate in the equilibrium system which he studied (2).

A factor that might contribute to Ca and P mobilization would be an increased ionic strength due to accumulation of metabolites in a closed system, but for such changes to cause a significant decrease in the activity coefficients of Ca and P in the medium and thus increase the apparent mobilization of these ions, they would have to be far greater than those observed here. The maximal change that could be attributed to ionic strength changes in these systems was one-eighth of the rise seen in Ca concentrations. Thus, changes in ionic strength do not appear to have contributed significantly to the phenomena observed.

The maintenance of higher Ca × P concentrations in the media surrounding living rather than dead bones is in agreement with the findings of others. Basset and Nordin found that human bones, killed by repeated freezing-thawing reached equilibrium with a lower Ca × P product than that of viable bones (5). The results are also supported by Raisz, Au and Tepperman, who recently reported higher Ca concentrations in the incubating fluids for living than for heat-inactivated bones, using weanling rats (16). All these results most probably are related to the presence of cell activity in viable samples. The findings strongly support the concept that cell activity is involved in the regulation of serum Ca × P levels, and is at least partly responsible for the supersaturation of normal serum.

The absence of glucose as a substrate for surviving bones resulted in a depression of Ca concentrations that approximated 50 per cent of the depression observed in heat-inactivated bones, and the bones killed owing to glucose starvation for 16 hours exhibited Ca values between these two groups. The reason that the depression in all three groups was not the same may be that in the glucose-deprived groups endogenous sources of carbohydrate, although very small, may have been utilized. In addition, metabolism of other endogenous substrates may have contributed. In any case, the results indicate that the rate of carbohydrate metabolism in the bone cells significantly affects the Ca concentrations that will be maintained in the surrounding fluids.

In the iodoacetate group the depression in Ca concentrations was significant but somewhat less

than in the glucose-deprived group. This might be explained partly by the increased ionic strength due to IA, and partly by metabolism above the block. However, the Ca × P products were the same in both groups, and the results suggest that the chief effects of glucose are dependent upon metabolism beyond triose phosphate dehydrogenase.

In the experiments in which the effects of glucose deprivation and iodoacetate were studied, a decrease in lactate of approximately 95 per cent was accompanied by a decrease in the Ca × P product of approximately 7 per cent. As appears from the experiments under anaerobic conditions, this simultaneous reduction does not mean that the product is directly influenced by the amount of lactate produced; rather, it suggests that the Ca × P product is diminished when metabolism, as expressed by the dwindling lactate production, is being slowed down.

On the basis of the results reported, a tentative diagram illustrating the components of the total Ca concentration in the incubating media surrounding bone from normal and parathyroid hormone-treated animals can be constructed as shown in Figure 4. For the control animals the fraction of the total Ca that can be assigned to passive solubility is 82 per cent, while 18 per cent can be considered the result of cellular activity. As can be seen, approximately one-half of this cellular "metabolic" fraction seems related to glucose metabolism and the remainder to other metabolic processes. In the parathyroid hormone-treated group the increment in total Ca concentration can be almost entirely attributed to increases in passive mineral solubility under the experimental conditions used here. However, although there is no apparent increase in the total size of the cellular "metabolic" component as compared with controls, the distribution between that which is glucose-dependent and that which is not has changed. The Ca-mobilizing effect related to glucose is enhanced, and the effect related to other metabolic phenomena is correspondingly reduced. A similar diagram for Ca × P products showed the same types of distribution.

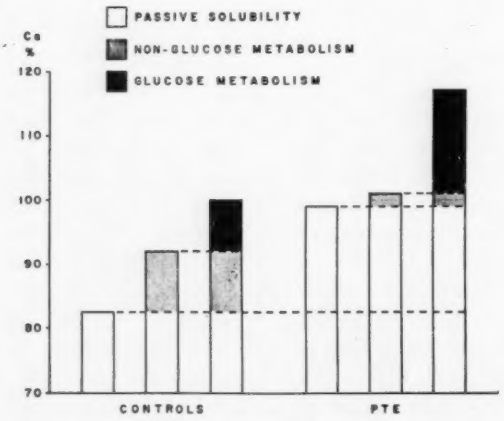

FIG. 4. COMPONENTS OF "STEADY STATE" CA CONCENTRATIONS IN THE MEDIA SURROUNDING BONES FROM NORMAL AND PTE-TREATED ANIMALS UNDER VARIOUS INCUBATING CONDITIONS. Data from Tables I and VI.

The significance of the modification of the pattern induced by the hormone is unknown; the mechanism by which the hormone increases the passive solubility of the mineral remains equally obscure, although it may be related to the increase in citrate content in dry bone which appears following treatment with parathyroid hormone (15, 19). As has been pointed out by Thompson (20), the presence of citrate in synthetic hydroxyapatite increases the relative solubility of such minerals. Another possibility might be that hydrogen ions from acid metabolites displace Ca ions from the crystal surface, as suggested by Neuman (2, 14). Such an effect on the solid phase would change the thermodynamic properties of the crystal and increase the solubility of the mineral (14).

SUMMARY

1. Mouse calvaria have been incubated *in vitro* until a steady state distribution of Ca and P between medium and sample was reached. These studies were designed to examine the relative importance of passive mineral solubility and various aspects of bone cellular metabolism in the maintenance of Ca and P concentrations in body fluids.

2. Inactivation of cellular activity by heat and by long-lasting starvation depressed Ca concentrations in the media 17.6 and 12.6 per cent, respectively. In surviving bone, glucose deprivation and iodoacetate (IA) inhibition decreased Ca 8.2 and 5.9 per cent, respectively, and lactate production about 94 per cent. These differences were all highly significant ($p < 0.001$). P concentrations, however, were roughly the same in all ex-

periments with surviving bones. While anaerobiasis increased lactate production 15.3 per cent, Ca was slightly decreased. The results support the concept that mobilization of Ca and P from bone mineral and the regulation of serum levels of these ions are dependent upon the rate and pattern of bone cell metabolism. Effects of glucose metabolism are dependent upon steps beyond triose phosphate dehydrogenase, but the rate of lactate production seems only indirectly involved.

3. Prior treatment with parathyroid extract (PTE) increased Ca 16.8 and lactate 22.3 per cent in the media surrounding surviving bones. The increment in Ca concentration due to the hormone treatment appeared to be the result of *a*) an increase in passive solubility of the mineral, and *b*) a modification of the pattern of cellular metabolic effects.

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STUDIES ON TISSUE UPTAKE OF INSULIN IN MAN USING A DIFFERENTIAL IMMUNOASSAY FOR ENDOGENOUS AND EXOGENOUS INSULIN *

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The radio-immunological technique for assaying endogenous plasma insulin in man recently described by Yalow and Berson (1, 2) promises to be of considerable value in the investigation of insulin metabolism. Other immunological methods that have been reported are not so sensitive as this method, since they fail to detect insulin in fasting normal human plasma (3). In contrast to the bioassay methods, which are limited to the estimation of "insulin-like" activity, the immunoassay is specific for insulin. The present communication describes a radio-immunological procedure for assaying endogenous (human) and exogenous (beef) insulin separately when both are present in human plasma. This differential assay is based on the observation that beef and human insulin react differently with antibeef insulin-binding antibodies in antisera from guinea pigs and man (1, 4). First, the plasma beef insulin level is measured with a relatively species-specific human antibeef insulin serum, which reacts poorly with human insulin. It is then possible to assay the plasma endogenous insulin with a guinea pig antibeef insulin serum which reacts strongly with both beef and human insulin. With this method it has been possible to demonstrate that, in man, both the liver and peripheral tissues remove endogenous and exogenous insulin from the blood.

METHODS

Hyperimmune antisera. Antibeef insulin antiserum was produced in guinea pigs by repeated weekly subcutaneous injections of Boots protamine zinc beef insulin (2). The human antibeef insulin antiserum used in this study was obtained from a diabetic patient who had always been treated with a Boots beef insulin preparation. Blood was drawn after insulin treatment had been withheld for 4 days.

Preparation of insulin-I^{131}. Since a minute amount of insulin-I^{131} serves as a tracer in the immunological system, it is essential to use a preparation with a high specific activity so that the counting rate may be adequate. Insulin-I^{131} with a specific activity of 300 to 600 mc per mg was obtained by the following method: 10 to 20 mc of carrier-free, thiosulphate-free I^{131} (as iodide) and 100 μl of iodine monochloride (5) are added simultaneously to 20 to 30 μg of Boots crystalline beef insulin in phosphate buffer, pH 7.3. After shaking for 3 minutes, 20 to 50 per cent of the mixture by volume is immediately dialyzed against distilled water at 0° C in order to remove free I^{131}. A variable amount of insulin-I^{131} is damaged by radiation; the undamaged insulin-I^{131} is recovered after separation from the damaged fraction on a cellulose column (2).

Assay of endogenous insulin in plasma. This assay has been described in detail by Yalow and Berson (2). Beef insulin-I^{131} in trace amounts is allowed to react with a suitable concentration of guinea pig antibeef insulin serum so that two-thirds to three-quarters of the beef insulin-I^{131} is bound to antibody. The bound insulin-I^{131} is separated from the free (unbound) insulin-I^{131} by chromatoelectrophoresis. The relative amounts of the bound and free fractions are recorded by a paper strip radioactivity scanner. Unlabeled human insulin competes with the beef insulin-I^{131} for binding by antibeef insulin antibody (1, 2) and causes a decrease in the ratio of bound insulin-I^{131}/free insulin-I^{131} (B/F ratio). The curvilinear decrease in the B/F ratio as the concentration of unlabeled insulin increases permits a quantitative assay of human insulin.

A crude human insulin powder [1] was used as the standard for human insulin. We have accepted Yalow and Berson's estimate of the pure insulin content of this crude preparation: 27.3 per cent pure insulin by weight or 6 U per mg (assuming 22 U per mg for pure insulin). Recently we have found that this estimate agrees satisfactorily with our own finding of 5.61 U per mg (SE, 0.58), using as a standard for comparison a pure human insulin.[2] These results are to be reported in full elsewhere.

All dilutions of insulin and antiserum were made in 0.1 ionic strength veronal buffer containing 0.25 per cent

* This work was supported by grants from the Medical Research Council of Great Britain and British Insulin Manufacturers.

[1] Kindly donated by Dr. A. M. Fisher, Connaught Laboratories, Toronto, Canada, to whom we express thanks.

[2] Kindly donated by Dr. P. Randle, Dept. of Biochemistry, University of Cambridge, England.

human serum albumin. The human serum albumin was tested for the presence of insulin and none was detected. Standard solutions with differing amounts of human insulin contained identical concentrations of beef insulin-I^{131} and antiserum. A standard curve is obtained by plotting the B/F ratio as a function of the concentration of added human insulin (calculated as "pure" human insulin after correction for damaged components of insulin-I^{131}) (Figure 1).

The assay of endogenous plasma insulin was accomplished by substituting the plasma sample for the human insulin. The B/F ratio given by the plasma sample is then referred to the standard curve for the corresponding insulin concentration.

Differential assay of beef and human insulin. Beef insulin reacts strongly with antibeef insulin antibodies in both guinea pig and human antisera. Human insulin reacts relatively strongly with antibeef insulin antibodies in guinea pig antisera but usually only weakly with antibodies in human antisera (1, 4, 6). When both beef and human insulin are present in the same mixture, human antibeef insulin serum is used to measure the beef insulin content of the mixture and guinea pig antibeef insulin serum to measure the human insulin.

A standard curve showing the B/F ratio plotted as a function of beef insulin concentration in human antiserum is shown in Figure 2. Compared with beef insulin, the effect of human insulin on the B/F ratio is almost negligible. This is in contrast to the standard curves obtained in the guinea pig antiserum (Figure 1) which is relatively sensitive to human insulin. These standard curves are plotted after a correction has been made for damaged components of insulin-I^{131} (2).

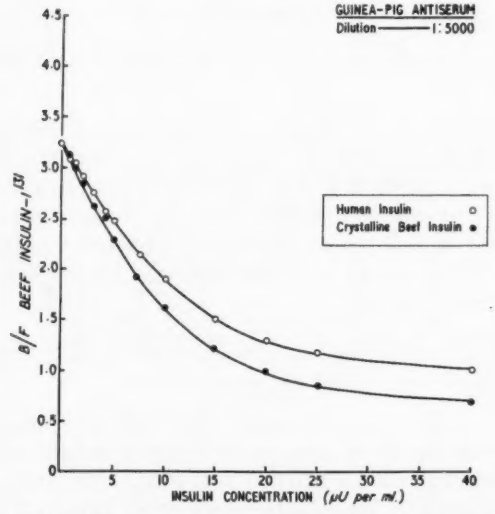

FIG. 1. STANDARD CURVES IN GUINEA-PIG ANTISERUM: RATIO, BOUND INSULIN I^{131}/FREE INSULIN I^{131} (B/F RATIO) AS A FUNCTION OF THE CONCENTRATIONS OF HUMAN INSULIN OR CRYSTALLINE BEEF INSULIN.

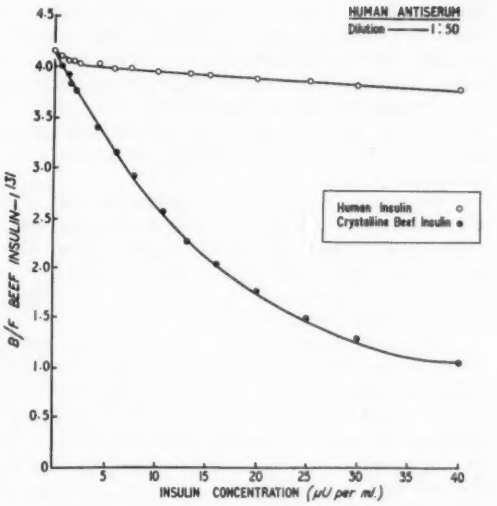

FIG. 2. STANDARD CURVES IN HUMAN ANTISERUM: B/F RATIO (BEEF INSULIN-I^{131}) AS A FUNCTION OF THE CONCENTRATION OF HUMAN INSULIN OR CRYSTALLINE BEEF INSULIN.

It should be possible to repeat the standard curves with identical B/F ratios and slopes if the same amount of radioactive insulin, unlabeled insulin and antibody are used every time. In practice, however, the adsorption of insulin to glassware and the variation in the proportion of damaged I^{131} after each iodination makes it difficult to ensure that the amount of tracer insulin-I^{131} is invariably constant. Thus, standard curves were similar but not identical, with beef insulin-I^{131} in a final concentration of 0.05 mμg per ml.

A mixture containing beef and human insulin is first incubated in human antiserum. The resultant B/F ratio is referred to the standard curve to give the beef insulin content of the mixture. The B/F ratio of the mixture is then determined in the guinea pig antiserum which is sensitive to both beef and human insulin. The human insulin is assayed in the guinea pig antiserum by determining the concentration of human insulin which must be added to the amount of beef insulin previously measured to give the B/F ratio of the unknown mixture. It was convenient to set up tubes containing the known amount of beef insulin plus known amounts of human insulin in guinea pig antiserum at the same time as the unknown mixture. Clearly, a small error in the estimation of beef insulin may cause a large error in the assay of human insulin in a mixture containing both insulins. As the initial steep slope of the standard curve is the most sensitive index of a small change in the insulin concentration, it is preferable to dilute mixtures containing a large amount of beef insulin to obtain a suitable B/F ratio.

Guinea pig antisera are usually less species specific than are human antisera, but the degree of species speci-

ficity varies in different antisera, whether human or guinea pig. For the purposes of differential assay it is desirable that species specificity should be maximal in human antiserum and minimal in guinea pig antiserum. Many human and guinea pig antisera were tested, and the particular antisera used in the present investigation came closest to meeting requirements regarding species specificity.

In the absence of beef insulin, endogenous plasma insulin was assayed against the standard curve for human insulin in the guinea pig antibeef insulin system (Figure 1). All plasma samples were diluted at least 1:10 to reduce incubation damage to a minimum (2), and all mixtures were incubated at 4° C for 4 days.

Collection of hepatic venous, femoral arterial and femoral venous blood. In patients with end-to-side portacaval anastomoses or blocked portal veins the portal blood flow is shunted into the systemic circulation. Blood enters the liver only through the hepatic artery and leaves via the hepatic veins. The balance of insulin across the liver can then be measured directly by the difference between arterial and hepatic venous plasma insulin levels. The hepatic uptake of insulin may be calculated as the product of the arteriohepatic venous plasma insulin difference and the estimated hepatic plasma flow. Seven patients were studied. Two (Ha and Ki) had surgical portacaval anastomoses and cirrhosis of the liver. Two (Ar and Ma) had blocked main portal veins without cirrhosis. Two (Co and Ja) had a portal collateral circulation secondary to cirrhosis or to infiltration of the liver with extramedullary hematopoeitic tissue. One patient (Br) had ascites due to a reticulosis but had no portal collateral circulation.

All patients were fasting and sedated. Blood samples were taken from a catheter deep in the hepatic vein and an indwelling catheter in the femoral artery and vein. Hepatic blood flow was estimated at 10-minute intervals by the clearance and extraction method of Bradley, Ingelfinger, Bradley and Currie (7), with indocyanine green as the extractable material (8). Midway between the sampling times for blood flow measurement, blood samples (4 to 6 ml) were drawn simultaneously and at a constant rate from the femoral artery and hepatic vein and, in some subjects, femoral vein. The blood was immediately placed in iced tubes containing heparin, and plasma was separated in a refrigerated centrifuge. The plasma samples were stored in a deep-freeze for insulin assay several months later.

Glucagon-free beef insulin [3] was administered to 5 patients by a Sigma constant-infusion pump through an indwelling catheter in a femoral vein at a rate of 0.07 to 0.1 U per kg body weight per hour. This rate of insulin infusion was chosen to produce a gradual modest decrease in in the arterial glucose concentration in order that the "counter-regulatory" responses to hypoglycemia might be kept to a minimum (9). One patient manifested the signs and symptoms of hypoglycemia during the infusion; the others tended to be lethargic. None of the subjects had previously received insulin.

[3] Burroughs-Wellcome glucagon-free crystalline beef insulin.

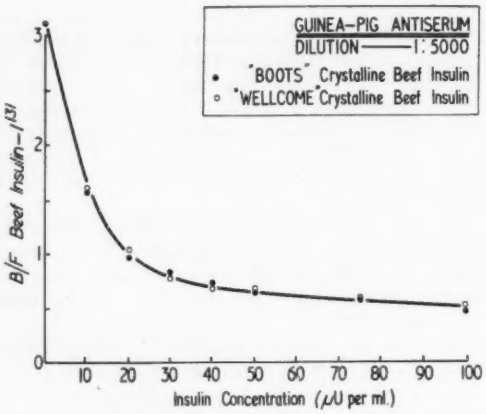

FIG. 3. B/F RATIOS OF BEEF INSULIN-I^{131} AS A FUNCTION OF THE CONCENTRATION OF BOOTS AND WELLCOME CRYSTALLINE BEEF INSULIN.

Comparison of different preparations of beef insulin. Boots 6-times recrystallized beef insulin, assayed at 22.2 U per mg, was used as the routine standard for beef insulin. Since Wellcome glucagon-free crystalline beef insulin, assayed at 22.4 U per mg, was the insulin infused into patients, the effect was compared of the two beef insulins on the B/F ratio for beef insulin-I^{131} (Figure 3). There is no significant difference between the standard curves for Boots insulin and Wellcome insulin.

RESULTS

1. *Normal fasting endogenous insulin concentrations*

The mean normal fasting endogenous insulin concentration of 30 normal subjects was 23 μU per ml (SD ± 17.4, range 0 to 70 μU per ml). This compares well with Yalow and Berson's mean values of 21 μU per ml (SD ± 15.4, range 0 to 66 μU per ml) for a similar number of normal subjects (2).

2. *Assay of model mixtures containing known amounts of beef and human insulin*

a) No plasma present. The differential assay was applied to various combinations of beef and human insulin which had been added to the radioactive beef insulin-antiserum systems (Figure 4). The recovery of beef insulin was virtually quantitative in all of the insulin combinations tested. The recovery of human insulin, good in mixtures

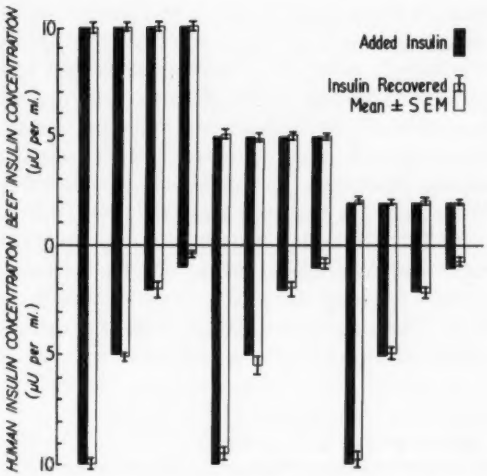

FIG. 4. RECOVERY OF BEEF AND HUMAN INSULIN WHEN BOTH INSULINS WERE ADDED *in vitro* TO THE BEEF INSULIN-I^{131}-ANTISERUM SYSTEMS. Each vertical column above and below the zero line shows the concentration of beef and human insulin, respectively, in a particular mixture. Mean values for insulin recovery (unshaded columns) were derived from four replicate determinations.

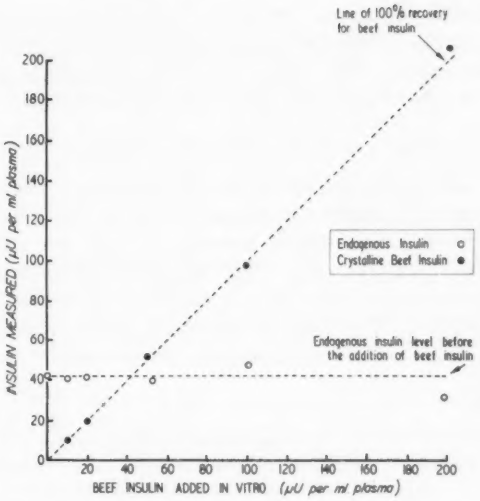

FIG. 5. ASSAY OF ENDOGENOUS INSULIN AND RECOVERY OF BEEF INSULIN AFTER ADDING BEEF INSULIN TO A FASTING PLASMA SAMPLE. The endogenous insulin concentration was 42 μU per ml before the addition of beef insulin.

containing the higher concentrations of human insulin, was less accurate when the amount of human insulin was small, particularly in the presence of the higher concentrations of beef insulin.

b) In plasma. Endogenous insulin was assayed in plasma from a normal fasting subject. The differential assay was then applied to a number of aliquots of this plasma after known amounts of beef insulin had been added *in vitro* (Figure 5). The recovery of beef insulin, in concentrations varying from 10 to 200 μU per ml plasma, was almost complete in the presence of an endogenous insulin content of 40 μU per ml plasma. Assay of endogenous insulin in plasma containing different beef insulin concentrations yielded rea-

TABLE I

Hepatic uptake of endogenous insulin *

Subject		Time in minutes 0	10	20	30	40	50	60	Mean
Ha, 42 years;	A	37	34	26	21	28	24	18	
80 kg;	HV	16	16	14	10	14	14	13	
portacaval	A − HV	21	18	12	11	14	8	5	
anastomosis;	(A − HV)/A	0.57	0.53	0.46	0.52	0.50	0.33	0.28	0.46
cirrhosis	EHPF			400	400	470	540	560	
	HIU per kg			60.0	55.0	82.2	54.0	35.0	57.2
Ar, 60 years;	A	22	22	24	22	24	26		
48 kg;	HV	16	17	21	18	20	22		
extrahepatic	A − HV	6	5	3	4	4	4		
portal block;	(A − HV)/A	0.27	0.23	0.12	0.18	0.17	0.15		0.19
liver normal	EHPF		580	580	578	623	645		
	HIU per kg		60.4	36.2	48.2	51.9	53.8		50.1

* A = arterial plasma insulin concentration in μU per ml; HV = hepatic venous plasma insulin concentration in μU per ml; A − HV = arterial minus hepatic venous insulin concentration in μU per ml; (A − HV)/A = arterial minus hepatic venous insulin concentration divided by arterial insulin concentration; EHPF = estimated hepatic plasma flow in ml per minute; HIU = hepatic insulin uptake in μU per minute per kg body weight.

sonably consistent results. The endogenous and beef insulin concentrations in this model are representative of the plasma levels in the subjects in our study before and after beef insulin infusion.

3. Hepatic uptake of endogenous insulin

a) In the absence of insulin infusion. There was a significant arteriohepatic venous difference in endogenous insulin levels in all four subjects (Ki, Ma, Ha, Ar) who had either portacaval anastomoses or portal vein thromboses (Tables I and II). The clearance of insulin by the liver in a single trans-hepatic passage was shown by the ratio: (arteriohepatic venous difference/arterial plasma insulin concentration) $[(A - HV)/A]$. The hepatic endogenous insulin uptake per kilogram body weight (HIU) in these four patients was calculated as the product of the arteriohepatic venous plasma insulin difference and the estimated hepatic blood flow and expressed as microunits per minute per kilogram body weight (Tables I and II). The endogenous insulin $(A - HV)/A$ ratio was determined for each batch of plasma samples collected every 10 minutes (Tables I and II). The mean $(A - HV)/A$ ratio for endogenous insulin was 0.46 in Subject Ha (over 60

TABLE II

Hepatic uptake of insulin before and during the slow infusion of beef insulin *

Subject	Minutes:	Preinfusion values −20	−10	0	Mean	Time during beef insulin infusion 10	20	30	40	50	Mean
A. Complete portal-systemic shunt						*0.10 U/kg/hr*					
Ki, 45 years; 60.1 kg; portacaval anastomosis; cirrhosis	A endog.	13	15	14		15	12	10	10		
	exog.					87	161	214	246		
	HV endog.	8	8	6		5	5	5	2		
	exog.					62	97	105	128		
	A − HV endog.	5	7	8		10	7	5	8		
	exog.					25	72	109	118		
	(A − HV)/A endog.	0.38	0.47	0.57	0.47	0.33	0.42	0.50	0.80		0.51
	exog.					0.29	0.43	0.51	0.49		0.42
	EHPF			259		271	272	258	295		
	HIU endog.			34.5		45.1	31.7	21.5	29.5		32.0
	Per kg exog.					112.7	325.8	467. 9	579.2		
						0.10 U/kg/hr					
Ma, 45 years; 80 kg; extrahepatic portal block; myeloid leukemia	A endog.	38	45	39		40	33	28	21	21	
	exog.					85	152	202	231	251	
	HV endog.	23	26	23		28	19	20	12	14	
	exog.					46	91	112	130	145	
	A − HV endog.	15	19	16		12	14	8	9	6	
	exog.					39	61	90	101	106	
	A − HV/A endog.	0.40	0.42	0.41	0.41	0.30	0.42	0.29	0.43	0.33	0.36
	exog.					0.46	0.40	0.44	0.43	0.42	0.43
	EHPF			820		820	820	820	820	820	
	HIU endog.			164.0		123.0	143.5	82.0	92.2	61.5	100.4
	Per kg exog.					399.8	625.2	922.5	1,035.2	1,086.5	
B. Partial portal-systemic shunt						*0.10 U/kg/hr*					
Co, 60 years; 60 kg; myelosclerosis	A endog.	48	49	46		30	10	0	0	0	
	exog.					74	149	198	246	281	
	HV endog.	56	54	50		30	16	0	0	0	
	exog.					47	85	152	183	209	
						0.07 U/kg/hr					
Ja, 42 years; 50 kg; cirrhosis	A endog.	8	7	8		5	5	0	0	0	
	exog.					88	157	221	253	262	
	HV endog.	18	12	12		15	12	5	0	0	
	exog.					59	109	165	208	212	
C. No portal-systemic shunt						*0.10 U/kg/hr*					
Br, 50 years; 80 kg; reticulosis	A endog.	18	11	14		18	16	13	12		
	exog.					58	96	152	192		
	HV endog.	35	32	35		38	29	31	26		
	exog.										

* Endog. =endogenous (human) insulin; exog. =exogenous (beef) insulin. See Table I for other abbreviations.

minutes) and 0.19 in Subject Ar (over 50 minutes), and in Subjects Ki and Ma over 30 minutes the ratios were 0.47 and 0.41, respectively. The average endogenous insulin (A − HV)/A ratio of 19 different determinations in the four patients (Ki, Ma, Ha, Ar) was 0.41. The mean endogenous HIU during a 50-minute period was 57 μU per minute per kg in Subject Ha and 50 μU in Subject Ar. In Subjects Ki and Ma the HIU values were 34 and 164 μU per minute per kg, respectively.

b) During insulin infusion. A hepatic uptake of endogenous insulin was still apparent during the infusion of beef insulin. The mean (A − HV)/A ratio for endogenous insulin was 0.51 in Subject Ki, with a HIU of 32 μU per minute per kg during a 40-minute infusion, and 0.36 in Subject Ma, with a HIU of 100 μU per minute per kg during a 50-minute infusion (Table II). The similarity of these ratios and HIU values to the respective preinfusion values suggests that the proportion of endogenous insulin cleared by the liver was not affected by the presence of exogenous insulin.

Of the three patients who had patent portal veins, Co and Ja had a collateral portal systemic circulation, but portal blood was flowing through the liver. All three patients showed higher levels of endogenous insulin in the hepatic venous samples than in arterial samples, both before and during insulin infusion.

4. Hepatic uptake of exogenous insulin

This may be compared with that for endogenous insulin in Table II. The (A − HV)/A ratios for endogenous insulin before and during the beef insulin infusion were remarkably similar to those for exogenous insulin in Patients Ma and Ki. The mean (A − HV)/A ratio for exogenous insulin was 0.42 in both Ma (4 determinations) and Ki (5 determinations). The (A − HV)/A ratio did not change significantly with rising arterial plasma insulin levels, nor was there any appreciable variation in estimated hepatic plasma flow. Thus, it appears that insulin was removed by the liver according to the law of a first-order reaction. The mechanism governing hepatic insulin capture was not saturated by arterial plasma insulin concentrations exceeding 250 μU per ml. While the hepatic insulin uptake cannot be calculated in the patients with intact portal veins, it is apparent that in these patients a significant amount of exogenous insulin was also removed from the splanchnic circulation (Table II) by the liver.

TABLE III

*Balance of endogenous insulin across the periphery; Subject Ha**

	Time in minutes						
	0	10	20	30	40	50	60
A	37	34	26	21	28	24	18
FV	30	29	20	16	22	20	16
A − FV	7	5	6	5	6	4	2
(A − FV)/A	0.19	0.15	0.23	0.24	0.21	0.17	0.11

* A = arterial plasma insulin concentration in μU per ml; FV = femoral venous plasma insulin concentration in μU per ml; A − FV = arterial minus femoral venous insulin concentration in μU per ml; (A − FV)/A = arterial minus femoral venous insulin concentration divided by arterial insulin concentration.

5. Peripheral insulin uptake

A definite femoral arteriovenous difference for both endogenous and exogenous insulin is shown in Tables III and IV. Only beef insulin was measured in plasma collected during the infusion. The peripheral (A − HV)/A ratio, which reflects the insulin uptake of the whole leg, comprising a number of different tissues, was much more variable than the (A − HV)/A ratio across the liver, which was also larger.

6. Velocity constant of elimination of exogenous insulin

The mean increase in arterial plasma total (endogenous plus exogenous) insulin concentration in seven patients during 50 minutes of beef insulin infusion is shown in Figure 6. The mean preinfusion arterial plasma endogenous insulin concentration was 25 μU per ml. It was not feasible to continue the beef insulin infusion for a longer period in an attempt to reach the steady state. Assumed that the elimination of the infused insulin from arterial plasma proceeds as a first-order reaction, sufficient data are available to estimate a hypothetical equilibrium concentration of plasma insulin (see Appendix); tentative values for the velocity constant of elimination, k, and the mean volume of distribution, v, could then be computed. The mean velocity constant of elimination, k, was 0.02 minute^{-1} and the mean volume of distribu-

TABLE IV

Peripheral uptake of insulin before and during the slow infusion of beef insulin *

Subjects	Minutes:	Endogenous insulin, preinfusion values			Beef insulin values during infusion				
		−20	−10	0	10	20	30	40	50
Ki	A	13	15	14	87	169	214	246	
	FV	11	12	10	81	161	188	202	
	A − FV	2	3	4	6	8	26	34	
	(A − FV)/A	0.15	0.20	0.28	0.07	0.05	0.12	0.14	
Ma	A	38	45	39	85	152	202	231	251
	FV	36	38	30	79	136	180	218	233
	A − FV	2	7	9	6	16	22	13	18
	(A − FV)/A	0.05	0.16	0.23	0.07	0.10	0.11	0.06	0.07
Br	A	18	11	14	58	96	152	192	
	FV	14	8	9	51	89	128	177	
	A − FV	4	3	5	7	7	24	15	
	(A − FV)/A	0.22	0.27	0.36	0.12	0.07	0.16	0.08	
Mean	A	23	24	22	77	139	189	223	
	FV	20	19	16	70	129	165	199	
	A − FV	3	5	6	7	10	24	24	
	(A − FV)/A	0.13	0.21	0.27	0.09	0.07	0.13	0.11	
Over-all mean	(A − FV)/A		0.20				0.10		

* See Table III for abbreviations.

tion, v, was 13 L, or 20 per cent of the mean body weight.

7. Effect of oxygenation and deoxygenation of whole blood

It is reported that oxygenation of blood either in the lungs or *in vitro* can lead to a rise in the

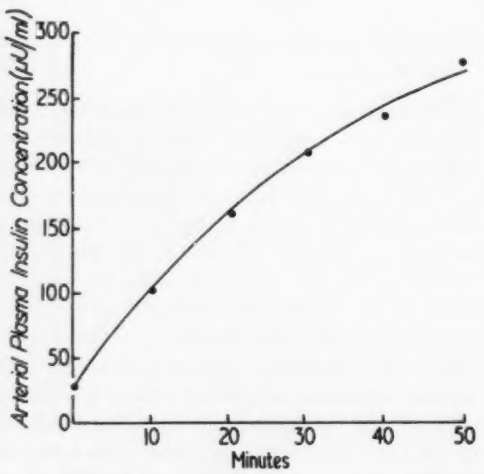

FIG. 6. MEAN RISE IN ARTERIAL PLASMA TOTAL INSULIN CONCENTRATION DURING THE INFUSION OF BEEF INSULIN. The mean arterial plasma endogenous insulin concentration before infusion was 25 μU per ml.

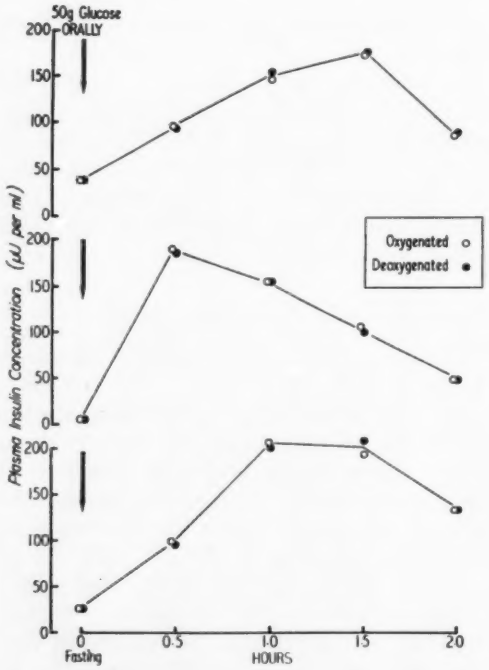

FIG. 7. PLASMA ENDOGENOUS INSULIN CONCENTRATIONS AFTER *in vitro* OXYGENATION AND DEOXYGENATION OF VENOUS WHOLE BLOOD. Taken during a glucose tolerance test in 3 normal subjects; each symbol represents the mean of duplicate determinations.

biologically estimated endogenous insulin-like activity (10, 11). A similar effect on the immunoassay would give rise to spurious arteriovenous differences in insulin levels. A wide range of endogenous insulin levels was obtained by drawing venous blood during a glucose tolerance test in three normal subjects. Plasma was immediately separated after *in vitro* oxygenation and deoxygenation of aliquots of whole blood (11). Immunoassay of plasma separated from the same sample of blood after oxygenation and deoxygenation showed no significant difference in the levels of endogenous insulin (Figure 7). Thus, arteriovenous differences in insulin concentration demonstrated by immunoassay could not be ascribed to the influence of oxygenation and deoxygenation.

DISCUSSION

Biological methods that measure "insulin-like" activity in plasma have given widely differing estimates of the normal range for the fasting plasma insulin concentration (12–18). Yalow and Berson have pointed out that the plasma insulin levels obtained by their immunoassay technique are in agreement with the lowest of these estimates (2). The fasting venous endogenous insulin concentrations in this study accord well with the findings of Yalow and Berson in normal subjects and cirrhotic patients, confirming the reproducibility and consistency of the radio-immunoassay technique.

The differential assay of endogenous and exogenous insulin when both are present in the same plasma is based upon the marked differences in species specificity that can be found in guinea pig and human antibeef insulin antisera. The differential assay provides a sensitive and precise measurement of beef insulin, independent of the concentration of human insulin in the insulin mixture. The measurement of human insulin, primarily dependent upon the complete recovery of beef insulin, is reliable at most concentrations of human insulin, but accuracy may be impaired in a mixture combining a very low human insulin content with a high beef insulin concentration. It has been suggested that differences and similarities in the binding of various animal insulins with antibodies in human antibeef-pork insulin sera may be due to differences in the amino acid sequence of residues 8 to 10 of the A-chain (4). We have no adequate explanation for the variations in species specificity shown by human or by guinea pig antibeef insulin sera. It should be noted that insulin preparations made by British manufacturers contain only beef insulin. In our experience different human antibeef insulin antisera have shown a wide range of species specificity for human and beef insulins. The degree of species specificity is, however, constant and reproducible for any particular antiserum using trace concentrations of insulin-I^{131}. Grodsky and Forsham recently reported minor or less than 3-fold differences in the binding of human and beef insulin in human antibeef-pork antisera (19), in contrast to the 25- to 100-fold differences described by Berson and Yalow (4, 6). This apparent discrepancy could be related to the variations in species specificity in different human antisera, or could be due to differences in technique. Cross reactions between animal insulins have been demonstrated in other immunological systems by Arquilla and Stavitsky (3) with the red cell hemagglutination-inhibition technique.

The potential application of the procedure described for assaying beef and human insulin, when both are present in plasma, may have limitations. Its use might be confined to patients who are receiving exogenous insulin for the first time or who have been treated with insulin for only a short while and have not developed antibodies to insulin. While it is possible to dilute insulin-treated diabetic plasma containing insulin-binding antibodies so that these have little effect *in vitro*, the reaction of insulin-binding antibodies with endogenous insulin *in vivo* and the effects of these antibodies on endogenous insulin degradation and distribution may invalidate the differential assay described.

It has been suggested that *in vitro* biological methods cannot be used to measure arteriovenous differences in insulin-like activity. The insulin-like activity of the blood in the venous drainage to the pancreas, measured by the rat diaphragm or the rat adipose tissue method, is less than that in simultaneously sampled arterial blood; and oxygenation of blood either in the lungs or *in vitro* can lead to a rise in the biologically estimated insulin-like activity (10, 11). It is unlikely that the arteriovenous differences in insulin concentration demonstrated by immunoassay could be ascribed to

the influence of oxygenation and deoxygenation. No significant difference has been found in the plasma insulin concentration of the blood after oxygenation and deoxygenation *in vitro*. The patients with intact portal veins showed a higher insulin concentration in hepatic venous plasma than in arterial plasma.

The results of this investigation show that, in man, the liver is capable of removing 20 to 50 per cent of the insulin passing through it for periods of over 1 hour. Mortimore and Tietze (20) found that about 40 per cent of insulin-I^{131} entering an isolated, cyclically perfused rat liver, was removed in a single trans-hepatic circulation. Madison, Combes, Unger and Kaplan compared the volume of distribution of insulin after a rapid injection into the portal vein (21) and calculated that more than 50 per cent of the insulin-I^{131} was bound to the liver in a single trans-hepatic circulation.

Little is known about the mechanism of capture of insulin by the liver. While it appears from our results that the hepatic uptake of insulin is a first-order reaction, there was no evidence of saturation of the sites of hepatic removal by the high level of insulin toward the end of the beef insulin infusion.

The proportion of insulin taken up by the liver before and during insulin infusion remained relatively constant in our patients. This may indicate that hepatic uptake was maximal throughout, with the arterial insulin concentration as the rate-limiting factor, but it should be noted that certain aspects in this investigation were distinctly not physiological: the physiological secretion of insulin is evoked by hyperglycemia, which was not present; a portal systemic shunt or liver disease was present in most of the patients investigated, and could result in findings that might not occur in healthy subjects. In spite of these considerations the radio-immunoassay method has demonstrated a hepatic and peripheral uptake of insulin in man. This technique may be a valuable means for investigating endogenous or exogenous insulin uptake by various tissues in patients with disorders of carbohydrate metabolism. Such a study might test whether some diabetics have defects in peripheral or hepatic metabolism of insulin. In some diabetics an abnormal insulin might be present that reacts well immunologically but poorly as a hormone *in vivo*. Immunoassay has already shown that absolute insulin deficiency is not the cause of hyperglycemia in early maturity onset diabetics (2, 22) and at least in some juvenile diabetics (22).

The present estimations of mean velocity constant of elimination and mean volume of distribution of insulin are based upon two theoretical considerations: *1*) the assumption that the infused insulin is eliminated as a first-order reaction, and *2*) formulae that have been constructed to express the elimination of a substance according to first-order kinetics during a constant intravenous infusion. Similar mathematical treatment has been used to estimate the velocity constant of elimination (k) and the volume of distribution (v) of glucose (23) and vasopressin (24) administered by constant intravenous infusion until a steady state supervened. The assumptions in the mathematical analysis are not free from objections (see Appendix). The meaning of the concept of a volume of distribution, v, as determined during a constant intravenous infusion, is complicated when a comparison is made with the method for obtaining v after a single intravenous injection of a substance. After a single injection, v is dependent upon the hypothetical concentration that would exist immediately after the injection if the steady state of infusion were reached at once, by extrapolation from the logarithmically linear part of the disappearance curve, ignoring the initial high concentration after the injection.

It has previously been shown that after a single intravenous injection of insulin-I^{131} in amounts ranging from 0.1 to 7.0 U, the degradation reaction follows first-order kinetics (25). The mean velocity constant of elimination, k, was 0.02 minute^{-1} and the mean apparent volume of distribution, v, was 37 per cent of body weight in about 45 minutes, distribution being about half completed at 15 minutes. These data are thus not comparable with the k value in the present study which is numerically equal (0.02 minute^{-1}) and a v value of 20 per cent of the mean body weight, since these values are derived from constant intravenous infusion data.

SUMMARY

1. Immunoassay of plasma insulin in man, as devised by Yalow and Berson, is confirmed to be

a highly sensitive method for insulin assay, yielding consistent and reproducible results. Normal fasting endogenous plasma insulin concentrations did not exceed 70 μU per ml.

2. A sensitive differential immunoassay is presented for measuring separately beef and human insulin in plasma in man after beef insulin administration. This assay is based upon differences in species specificity shown by human and guinea pig antibeef insulin antisera.

3. Immunoassay, in contrast to the rat adipose tissue or diaphragm methods, is not influenced by oxygenation or deoxygenation of whole blood *in vitro* or *in vivo*. This procedure may therefore be used to measure arteriovenous differences in insulin concentration.

4. A significant hepatic uptake of endogenous insulin is demonstrated in four patients with portacaval anastomoses or extrahepatic portal blocks. Sampling of arterial and hepatovenous blood and estimation of hepatic blood flow permits the calculation of the balance of insulin across the liver. The mean proportion of arterial insulin removed by the liver, reflected by the ratio: (arteriohepatic venous difference in insulin concentration/arterial insulin concentration) [(A − HV)/A] was similar for endogenous (mean, 0.40) and exogenous (mean, 0.42) insulin. The hepatic uptake of endogenous insulin in each patient was relatively constant over a period of 60 minutes. During beef insulin infusion the mechanism for beef insulin capture in the liver behaved as though governed by a first-order system, and was not saturated by arterial plasma beef insulin levels of 250 μU per ml.

5. A definite peripheral insulin uptake of endogenous and exogenous insulin was demonstrated. The (A − V)/A ratios are smaller and more variable than the equivalent ratios across the liver.

6. The calculated mean velocity constant of elimination (k) for the infused insulin was 0.02 minute^{-1}.

APPENDIX

In a first-order reaction the infused substance is eliminated at a rate proportional to the amount of the substance present in the plasma. The linear equation expressing this state is:

$$\frac{dc}{dt} + kc = \frac{p}{v}$$

where c = concentration of insulin in plasma, p = rate of infusion which is known and constant, v = volume of distribution which is assumed to be constant, and k = velocity constant of elimination. If $c = c_1$ when $t = 0$, integration yields:

$$c = \frac{p}{kv} - \frac{(p - kvc_1)}{kv} e^{-k(t-t_0)}$$

and if $c = 0$ when $t = 0$, then:

$$c = \frac{p}{kv}(1 - e^{-kt})$$

The hypothetical equilibrium concentration, C_2 (the y asymptote), was estimated as the value that yielded a straight line when log $(C_2 - C)$ was plotted against time. The best fit for C_2 was 350 μU insulin per ml when C was regarded as the rise in plasma insulin concentration above the mean preinfusion level of 25 μU per ml.

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STIMULATION OF ACTIVE SODIUM TRANSPORT BY THE ISOLATED TOAD BLADDER WITH ALDOSTERONE *IN VITRO* *

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The tubular reabsorption of sodium in the mammalian kidney is stimulated after administration of aldosterone (1–3). The transport of sodium from the tubular lumen to the interstitial compartments, across the cells lining the renal tubule, represents an active process (4, 5), and one is therefore led to conclude that this process is influenced by aldosterone. However, the failure to observe a sodium retention confined to the treated side after injection of aldosterone into one renal artery of the normal dog (3) could cast doubt that aldosterone is the principle immediately responsible for renal sodium retention. The time lag of 1 to 2 hours regularly observed between intravenous administration of aldosterone and the first changes in sodium excretion by the kidney (6–8) is likewise intriguing.

The isolated toad bladder was thought suitable for a more direct examination of the stimulation of sodium reabsorption by aldosterone. This preparation consists of a single layer of mucosal cells supported by thin connective tissue in which blood vessels and isolated bundles of smooth muscle are interspersed; a layer of serosal cells separates the bladder from the peritoneal cavity. This simple biological membrane is capable of moving sodium actively from its mucosal to its serosal border (9), a process that can be conveniently followed by the short-circuit technique of Ussing and Zerahn (10). These investigators have shown that, when an external potential is applied so as to nullify the spontaneous transmembrane potential, the current measured in the external circuit is the quantitative expression of active sodium transport.

Previous experiments have established that aldosterone is a physiological hormone for the toad, *Bufo marinus*, and that sodium transport by the toad urinary bladder is stimulated after injection of the steroid into the animal (11). For the present studies the membrane was exposed to aldosterone *in vitro*, and stimulation of active sodium transport ensued.

MATERIAL AND METHODS

All toads (*Bufo marinus*, male and female) were maintained half-immersed in distilled water for 3 to 5 days before sacrifice, except for half of those used in the first experiment. The animals were pithed and both half-bladders were mounted in rapid succession between Lucite chambers (20 mm ID) as described by Ussing and Zerahn (10), with the membrane bathing in 8 to 10 ml aerated frog Ringer solution (Na, 113.5 mEq per L; K, 1.88 mEq per L; HCO_3, 2.38 mEq per L; Ca, 0.89 mmole per L; Cl, 114.9 mEq per L; osmolality, 220 mOsm per kg water; pH, 8.1 at room temperature after equilibration with air). The serosal surface of one half-bladder was exposed to frog Ringer solution containing *d,l*-aldosterone in concentrations ranging from 10 to 180 μg per 100 ml; i.e., 1.4×10^{-7} M to 2.5×10^{-6} M biologically active *d*-aldosterone. Measurements of short-circuit current and of DC resistance were made at 15- to 30-minute intervals for 3 to 4 hours. The DC resistance of the membrane was derived from the potential generated by imposing a current of 100 μa across the short-circuited preparation. In some experiments 1 U vasopressin (Pitressin; Parke, Davis) was added to the fluid bathing the serosal surface of the membrane after 180 to 210 minutes of incubation; short-circuit current was measured at 5-minute intervals during the following 20 to 30 minutes. In a series of 8 experiments, studies were performed on the short-circuited membrane, using Na^{24} and Na^{22} to measure the two unidirectional sodium fluxes simultaneously (9). Bladder tissue was analyzed for water, sodium, potassium, and chloride content as previ-

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ously described (11). Tissue glycogen concentration was determined by the anthrone method (12), after alkaline digestion of tissue samples stored frozen after incubation and weighing.

In some experiments 20 μg *d,l*-aldosterone 21-monoacetate (dissolved in 0.02 ml ethanol diluted to 0.1 ml with frog Ringer solution) was injected into the toads 24 and 4 hours before sacrifice. In another experimental series, 1 mg deoxycorticosterone acetate (Schering sublingual tablets dissolved in 0.5 ml frog Ringer solution) was injected into each animal daily for 5 days, with a last injection 4 hours before sacrifice.

RESULTS

1. In an initial series of experiments, paired half-bladders were obtained from 16 toads, 8 of which had been maintained in distilled water (water toads) and an equal number in saline (125 mEq Na per L: saline toads) for 3 to 5 days. For each experiment one half-bladder served as a control, while the solution bathing the serosal surface of the corresponding half-bladder contained 2.8×10^{-7} M *d*-aldosterone. Incubation was carried out for 4 hours. As seen in Figure 1 the mean short-circuit current across the membranes exposed to aldosterone started to increase above the control values after approximately 60 minutes. The stimulation due to aldosterone became increasingly apparent with time. For the water toads during the last hour of incubation, the short-circuit current averaged 90.8 ± 9.2 μa per 3.14 cm² as compared with a mean value of 37.7 ± 3.5 μa per 3.14 cm² for the paired control half-bladders ($p < 0.001$). With the saline toads mean values were 42.6 ± 8.5 μa per 3.14 cm² and 19.9 ± 3.6 μa per 3.14 cm², respectively ($p < 0.02$). Relatively and absolutely, the stimulation by aldosterone of active sodium transport across the toad bladder was more marked in the case of the water toads. Therefore, water toads were used for all subsequent studies.

Experiments with paired bladder halves were carried out to minimize the variability of the aldosterone effect on active sodium transport by the isolated bladder from toad to toad. In addition, each membrane was used as its own control by expressing the short-circuit current during the third hour of incubation, when the effect of aldosterone was quite pronounced, relative to the short-circuit current during the first hour of incubation, prior to a discernible hormonal effect. This form of presentation of the results seems justifiable because of the absence of a close correlation between sodium transport activity during the first hour of

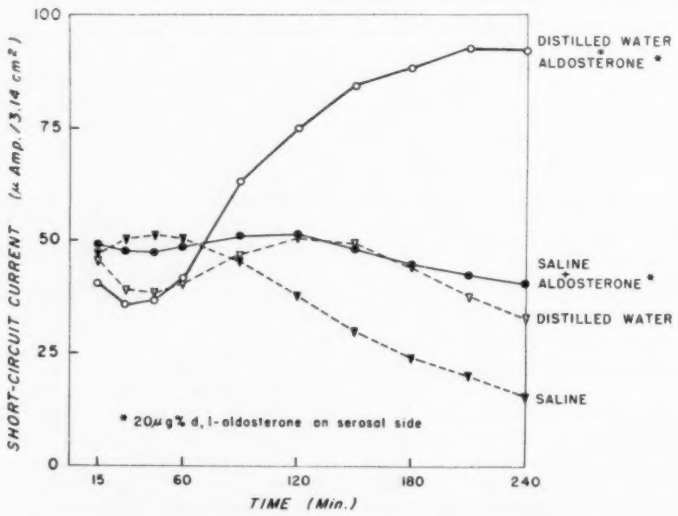

FIG. 1. STIMULATION BY ALDOSTERONE OF ACTIVE SODIUM TRANSPORT ACROSS THE TOAD BLADDER *in vitro*. Experiments performed on paired membranes. The serosal surface of one bladder half was exposed to aldosterone and the corresponding half served as control. Eight toads had been maintained in distilled water, eight in saline, prior to these incubations.

TABLE I

Water, sodium, potassium, and chloride content of toad bladder tissue after stimulation of sodium transport by aldosterone in vitro *

	Water	Sodium	Potassium	Chloride
	μl/100 mg dry residue		*μEq/g dry residue*	
Control tissue	381.6 ± 10.2	404.4 ± 11.9	299.8 ± 12.3	366.3 ± 13.1
Tissue exposed to aldosterone	367.7 ± 14.3	388.2 ± 12.5	291.0 ± 11.6	366.0 ± 14.1
			μEq/g tissue water	
Control tissue		107.4 ± 4.9	79.2 ± 2.7	96.7 ± 3.7
Tissue exposed to aldosterone		108.3 ± 6.1	80.5 ± 4.1	100.0 ± 2.5

* Means ± standard error of the means (SEM); each value represents the mean of 16 determinations; none of the differences is significant below the 40% level.

incubation and the spontaneous changes in sodium transport activity from the first to the third hour ($R = 0.58$ for 98 untreated membranes).

2. After the incubation just described, bladder tissue was analyzed for water, sodium, potassium, and chloride contents. Values were grouped according to the experimental conditions *in vitro* (Table I). Although the half-bladders exposed to aldosterone transported more than twice as much sodium as their paired controls in the hour preceding the end of the incubation, no significant difference was detectable in tissue water and electrolyte composition.

3. The validity of the short-circuit current as a quantitative expression of net sodium transport during stimulation of the half-bladders by aldosterone *in vitro* was examined in another series of experiments. Bidirectional sodium flux determinations were carried out for five consecutive periods of 30 minutes across eight short-circuited toad bladders, the serosal surface of which was exposed to 2.8×10^{-7} M *d*-aldosterone. From the results summarized in Table II, it is apparent that the assumption is valid. The agreement between both types of measurement was as good during the entire period of aldosterone effect, from minute 60 on, as during the period of steady state (stable short-circuit current) extending through the last hour of incubation. The mean ratios given in the last column of Table II are an expression of this agreement for the individual 30-minute periods.

4. The stimulation of active sodium transport under the influence of aldosterone requires the expenditure of additional energy by the cells concerned. Leaf and Dempsey (13) have shown that increased glycolysis is associated with a stimulation of sodium transport after addition of vasopressin to the preparation under aerobic conditions. Glycogen was therefore measured in 15 pairs of pooled membranes, each pool containing 4 to 8 toad half-bladders. As seen in Table III there was a decrease in tissue glycogen concentration in eight of nine paired experiments for which sodium transport was known to have been stimulated by aldosterone. For Experiment 292, such a stimulation was merely assumed.

TABLE II

Agreement between net sodium transport and short-circuit current measurements in the isolated toad bladder exposed to aldosterone

Minutes	Periods of observation of 30 min	Short-circuit current (*μa/3.14 cm²±SEM*)		Ratio derived
		Observed	Derived from sodium flux*	observed (% ±SEM)
Total 60 to 210	39	71.5 ± 3.24	71.3 ± 5.48	98.4 ± 5.5
During steady state 150 to 210	16	79.8 ± 4.1	80.6 ± 8.7	100.7 ± 8.4

* A current of 1 μa is generated by a net flux of 1.865×10^{-2} μEq sodium per 30 minutes.

TABLE III

Tissue glycogen content of the toad bladder after exposure to aldosterone in vitro *

Pool no.	Control	+Aldosterone
	A. Stimulation of sodium transport by aldosterone	
190	589.7	580.2
210	636.4	543.8
230	890.0	633.1
260	569.1	516.2
240	562.3	610.8
270	671.6	474.4
112	500.0	252.3
280	382.3	371.3
290	471.3	408.5
292	301.7	359.6
Mean	557.4	475.0
Mean difference ± SE mean difference: − 82.4 ± 36.4 ($p < 0.05$)		
	B. No stimulation of sodium transport by aldosterone	
130	488.3	539.8
140	393.4	419.9
310	297.6	336.7
330	351.5	344.7
120	221.0	210.5
Mean	350.4	370.3
Mean difference ± SE mean difference: + 19.9 ± 8.0 ($p > 0.05$)		

* μg/100 mg wet tissue weight.

The action of aldosterone on the metabolism of the preparation is linked to the hormonal stimulation of active sodium transport. This is demonstrated in the second part of Table III by the absence of change in tissue glycogen concentration when sodium transport was not influenced by aldosterone—owing to concentrations of hormone insufficient to bring about a stimulation of sodium transport; to the presence of spirolactone that inhibited the effect of aldosterone on sodium transport (14); or to incubation of the membrane in a potassium-free medium in which no sodium transport takes place.

5. An attempt has been made to establish whether there is a quantitative relationship between the extent of the stimulation of active sodium transport by aldosterone and the concentration of the latter in the fluid bathing the serosal surface of the membrane. The results of this study are presented in Table IV. An adequate appraisal of such relationship is hampered by the variable activity of sodium transport across the toad bladder, as already mentioned. No effect of aldosterone on sodium transport could be detected with a concentration of hormone of 1.4×10^{-7} M even when the values were compared with those obtained with the paired controls (eight experiments). On the other hand, in paired experiments a stimulation of sodium transport was noticeable with a concentration of 2.0×10^{-7} M *d*-aldosterone. With larger concentrations there was generally a greater effect on sodium transport. The duration of the latent period, approximately 1 hour, was not influenced by these changes in hormone concentration.

6. The responsiveness of active sodium transport to aldosterone *in vitro* appears to be dependent upon the preconditioning of the toad. It has already been mentioned that the response of the isolated toad bladder to aldosterone *in vitro* was smaller with saline toads than with water toads. Plasma aldosterone concentration is lower in the saline than in water toads (11). When the

TABLE IV

Stimulation of active sodium transport by the isolated toad bladder as a function of the concentration of aldosterone

No. of expts.			Short-circuit current (μa/3.14 cm²) means ± SEM		Increase from first to third hr (% ± propagated SEM)
			First hour	Third hour	
98		Control	56.26 ± 2.36	86.63 ± 3.30	167.2 ± 5.7
8	*d*-Aldosterone	1.4×10^{-7}M	40.7 ± 4.9	52.0 ± 8.9	121.3 ± 20.8
15		2.0×10^{-7}M	80.3 ± 6.6	115.0 ± 4.7	173.4 ± 9.2
8		2.4×10^{-7}M	54.4 ± 4.9	124.5 ± 18.5	215.0 ± 17.4
52		2.8×10^{-7}M	41.0 ± 1.5	91.0 ± 3.4	241.1 ± 5.3
18		4.0×10^{-7}M	62.8 ± 5.5	142.8 ± 12.5	247.4 ± 12.3
12		5.6×10^{-7}M	41.7 ± 5.2	115.4 ± 10.3	317.0 ± 15.3
12		10^{-6}M	60.3 ± 6.1	134.1 ± 12.8	242.3 ± 13.9
12		2.5×10^{-6}M	30.6 ± 4.0	101.1 ± 10.2	362.4 ± 16.5

TABLE V

Effect of aldosterone on active sodium transport by the isolated toad bladder; influence of the state of the process prior to experimental treatment

Preparation	No. paired expts.		Short-circuit current (μa/3.14 cm^2) means ± SEM		Increase, first to third hr (% ± propagated SEM)	Signif. of difference between control and treated paired membranes
			First hour	Third hour		
Saline, 5 days	8	C*	49.3 ± 6.2	30.6 ± 5.5	54.4 ± 21.9	p >0.2
		A	48.4 ± 5.5	48.1 ± 8.3	81.3 ± 20.7	
Distilled water, 3 days	8	C	42.0 ± 2.4	47.9 ± 3.6	113.7 ± 9.4	p <0.02
		A	39.7 ± 3.3	82.5 ± 7.5	220.6 ± 12.3	
Distilled water, 3 days: inject. aldosterone 24 and 4 hours before sacrifice	6	C	148.1 ± 7.1	172.3 ± 10.3	118.0 ± 7.7	p >0.7
		A	136.0 ± 13.3	150.7 ± 7.4	121.8 ± 10.9	
Distilled water, 5 days: daily injec. deoxycorticosterone	6	C	108.6 ± 10.7	100.0 ± 6.2	95.5 ± 11.6	p >0.5
		A	119.4 ± 8.9	100.0 ± 5.2	90.8 ± 9.1	

* C = control half-bladders. A = paired half-bladders; serosal surface was exposed to frog Ringer solution containing 2.8×10^{-7} M *d*-aldosterone.

activity of sodium transport by the bladder was enhanced by injection of aldosterone into water toads the day prior to the incubations, an effect of aldosterone added to the membrane *in vitro* could no longer be demonstrated (Table V). The same observation was made after injection of deoxycorticosterone acetate daily for 5 days, which also results in a conspicuous increase of sodium transport by the preparation, as shown at the bottom of the first column of Table V.

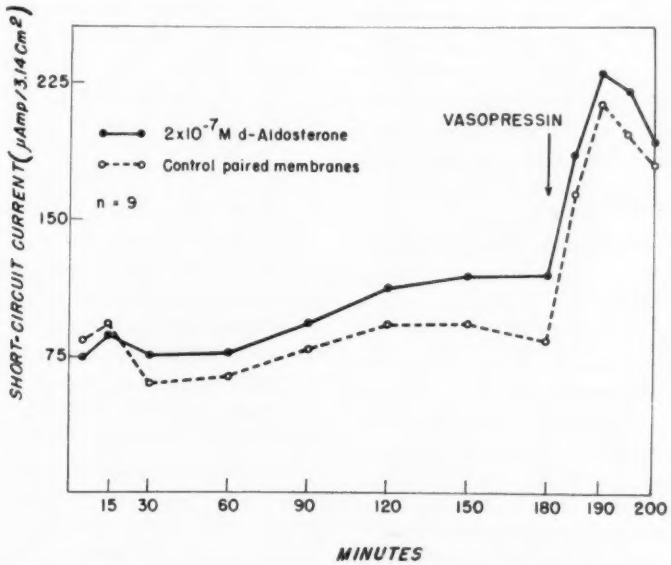

FIG. 2. THIS SERIES OF NINE PAIRED EXPERIMENTS ILLUSTRATES THE ADDITIVE CHARACTER OF THE RESPONSE OF NET SODIUM TRANSPORT TO ALDOSTERONE AND TO VASOPRESSIN.

7. Under the influence of aldosterone, the transmembrane potential increased almost proportionately to the hormonal effect on active sodium transport. Indeed, in 50 experiments the mean electric resistance of the untreated bladder half was 441 ± 15.8 ohms per 3.14 cm^2 during the first hour of incubation, and 454 ± 14.8 ohms per 3.14 cm^2 during the third hour. For the paired membranes exposed to aldosterone, the values averaged 414 ± 14.5 and 393 ± 12.5 ohms per 3.14 cm^2, respectively.

8. Active sodium transport by membranes stimulated by aldosterone still exhibited the usual responsiveness to vasopressin. When aldosterone was used in nine paired experiments at a concentration of 2×10^{-7} M, the short-circuit current during the third hour of incubation averaged 90.9 ± 5.9 μa per 3.14 cm^2 for the control half-bladders, rising to a mean of 189.9 μa during the 20-minute period that followed addition of vasopressin. For the paired half-bladders exposed to aldosterone, the mean values were 117.8 ± 6.6 and 211.1 μa per 3.14 cm^2, respectively (Figure 2). Thus the increase of short-circuit current was almost exactly the same for both groups of bladder halves. The additive character of the response of sodium transport to vasopressin and aldosterone has already been noticed after stimulation of sodium transport by injection of aldosterone into toads (11). When sodium transport was stimulated by injection of sodium-retaining steroid into water toads, a brisk response of sodium transport was still obtained after addition of vasopressin, even though these membranes no longer responded to aldosterone *in vitro* (Table V).

9. In 12 paired experiments deoxycorticosterone was added to the solution bathing the serosal surface of the toad bladder in concentrations of 2.0 and 4.0×10^{-5} M in 0.1 per cent ethanol (vol/vol). Although the latter are 200 times higher than effective concentrations of aldosterone, no effect on sodium transport could be observed despite an incubation lasting more than 4 hours (Table VI). The amounts of ethanol used to dissolve deoxycorticosterone do not modify the responsiveness of the preparation toward aldosterone.

TABLE VI

The influence of deoxycorticosterone on active sodium transport across the toad bladder in vitro

No. paired expts.		Short-circuit current (μa/3.14 cm^2) means ± SEM		Increase, first to third hr (% ± propagated SEM)
		First hour	Third hour	
12	C*	35.4 ± 3.4	58.5 ± 5.5	168.2 ± 13.4
	DOC	33.9 ± 2.8	51.3 ± 4.4	159.0 ± 11.9

* C = control half-bladders. DOC = paired half-bladders whose serosal surface was exposed to frog Ringer solution containing 2.0×10^{-5} M deoxycorticosterone in 6 experiments and 4.0×10^{-5} M deoxycoticosterone in 6 experiments.

DISCUSSION

These results demonstrate that active sodium transport can be stimulated by aldosterone *in vitro*. Concentrations of hormone less than seven times those found in the circulating blood of toads maintained in distilled water (11) were sufficient to elicit a significant elevation of the short-circuit current. The latter was shown to represent net sodium transport quantitatively. The equivalence between net sodium transport and short-circuit current was discovered by Ussing and Zerahn with the isolated frog skin (10), and it applies to the toad bladder preparation under baseline conditions (9) as well as after addition of vasopressin (13).

Active sodium transport requires the expenditure of metabolic energy by the cells concerned. Leaf, Dempsey, Page and Anderson have shown that, in the toad bladder, glycolysis represents a source of energy used for sodium transport (13, 15). The observed increase in glycolysis resulting from the stimulation of sodium transport by aldosterone was therefore not surprising.

The mode and exact site of action of aldosterone on sodium transport are not elucidated. A stimulation of sodium transport by the hormone was not accompanied by measurable changes in bladder tissue water, sodium, potassium, or chloride concentrations. Such negative observations have been made previously in studies of the toad bladder (11) and of rat kidney slices (16). Changes in the activity of sodium transport across specialized cells, such as the mucosal cells of the toad bladder and the cells of the renal tubule, need not be reflected in gross alterations of their ionic composition. Measurements of the intracellular sodium pool concerned with sodium transport would un-

doubtedly provide more meaningful information (17). On the other hand, in resting muscle cells, for instance, the active process is represented by an extrusion of sodium that balances the passive penetration of this ion across the cell membrane. After injection of aldosterone into mice, muscle sodium was actually decreased (18), presumably as the result of stimulation of the active process responsible for sodium extrusion.

Despite the variable pattern of sodium transport from one membrane to the other, as a rule an increased degree of stimulation of sodium transport resulted from incubation of the toad bladder with increasing concentrations of aldosterone. This was to be expected, since such a relationship forms the basis for measurement of aldosterone by bioassay (19–22). It is noteworthy that, as was the case with the toad bladder, the bioassay data failed to demonstrate a shortening of the latent period between administration of the steroid and the onset of sodium retention when larger amounts of aldosterone were used. This constitutes a strong argument that the latent period is not merely due to a slow build-up of the appropriate hormone concentration within the cell. A possibility is that aldosterone stimulates the synthesis of a substance involved in active sodium transport. This would explain the latent period and the dependency of the aldosterone action *in vitro* on the underlying activity of the sodium transport process as influenced by aldosterone secreted by or injected into the toad. Aldosterone *in vitro* had little effect on sodium transport when the adrenal secretion of aldosterone was low (saline toads). Immersion in distilled water, which results in a stimulation of the endogenous secretion of aldosterone, increased the responsiveness of the toad bladder to aldosterone *in vitro*. But, if sodium transport by the membrane was stimulated further, by a combination of a distilled water habitat and injection of a sodium-retaining steroid, aldosterone was devoid of measurable effect on sodium transport *in vitro*. Such observations confirm the studies made *in vivo* (11) and suggest that the hormone exerts its influence on a rate-limiting process.

The stimulation of sodium transport upon addition of vasopressin is added to that of aldosterone, and it is still demonstrable when aldosterone *in vitro* fails to influence sodium transport. Such independence suggests that the mechanism of action of both hormones on active sodium transport is different.

The complete lack of effect of deoxycorticosterone on sodium transport by the toad bladder *in vitro* merely confirms negative results obtained in the past with the isolated frog skin (23, 24). This ineffectiveness is intriguing because injection of the steroid into the toad brought about a large stimulation of sodium transport by the membrane, as shown in Table V.

In the case of the toad urinary bladder, active sodium reabsorption, whether stimulated by aldosterone or not, is not linked to any measurable extent to potassium or hydrogen secretion, as indicated by the agreement between sodium flux and short-circuit current measurements. It is tempting to assume that, in the mammalian nephron also, aldosterone exerts its influence primarily upon active sodium transport. The increased secretion of potassium and hydrogen ions by the distal portion of the renal tubule after administration of aldosterone (6) could then be regarded as a mere consequence of the increased transtubular potential generated by the hormonal stimulation of sodium transport. The transmembrane potential of the toad bladder was higher when sodium transport was stimulated by aldosterone. In rats likewise, the distal transtubular potential is elevated during sodium depletion (25), a situation producing a hypersecretion of aldosterone by the adrenal cortex (6).

SUMMARY

Active sodium transport by the isolated toad bladder was stimulated by aldosterone used *in vitro* at concentrations of 2.0 to 25×10^{-7} M, in frog Ringer solution. This stimulation became apparent after a latent period of approximately 1 hour, and it was accompanied by an increase in the transmembrane potential. The effect of the steroid hormone on sodium transport was a function of its concentration as well as of the state of the animal prior to the experiments.

The stimulation of sodium transport under the influence of aldosterone was associated with an increased glycolysis. It did not modify the responsiveness of the preparation to vasopressin.

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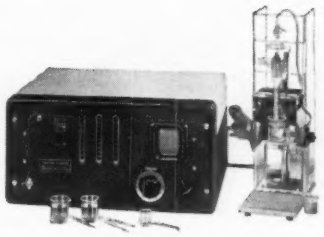

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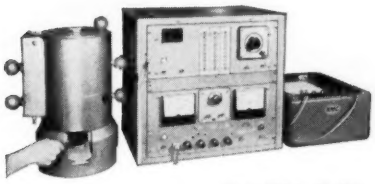

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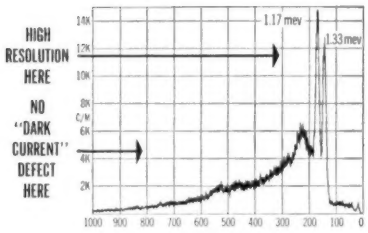

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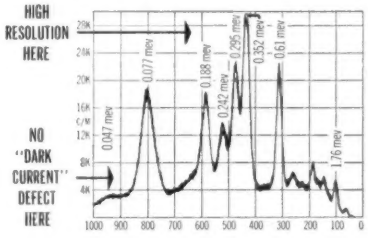

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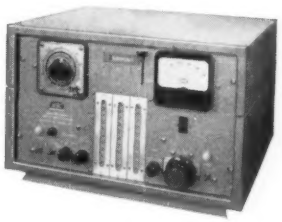

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